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Validation of Acute Traumatic Coagulopathy in an ovine model of trauma and haemorrhage

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Abstract

Perturbations in coagulation function are common following trauma and are independently associated with poor clinical outcomes. Trauma induced coagulopathy (TIC) was traditionally considered an iatrogenic process, attributed to the loss, dilution or dysfunction of coagulation proteases. It is now recognised that an acute endogenous coagulation dysfunction develops prior to medical intervention in response to a combination of tissue injury and hypoperfusion. This acute traumatic coagulopathy (ATC) is still defined clinically using traditional assays of coagulation function as a 20% increase in international normalised ratio (INR). Efforts have been made to characterise ATC using viscoelastic point of care testing; however there is no current universally accepted viscoelastic definition.

The pathogenesis of ATC remains poorly understood. Activation of the protein C pathway, fibrinolysis, platelet dysfunction and endothelial glycocalyx shedding are all hypothesised to play a role in development. However the exact contributions are still unknown and this current knowledge gap is impeding the development of effective and tailored resuscitation strategies for this subset of patients.

Pre-clinical animal research is a necessary adjunct for improving the understanding and management of ATC. Despite considerable interest in developing animal models of ATC there are few clinically relevant models that reflect the contemporary understanding of the condition. The development of a well-designed animal model of trauma may improve mechanistic understanding of ATC and facilitate the development of targeted treatment strategies.

This thesis describes an ovine model of complex trauma and haemorrhage that demonstrates coagulation changes using both traditional plasma based assays and point of care viscoelastic assays that are consistent with current definitions of ATC. The degree of coagulopathy was correlated with the degree of shock as quantified by arterial lactate. Coagulopathy was also associated with activation of the protein C pathway and shedding of the endothelial glycocalyx. Fibrinolysis did not make a significant contribution to the coagulopathy observed and there was no evidence of altered platelet function in this model.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

van Zyl N, Reade MC, Fraser JF. *Experimental animal models of traumatic coagulopathy: A systematic review.* Shock 2015; 44(1):16-24

van Zyl N, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SG, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and hemorrhage*. J Trauma Acute Care Surg 2016; 81(4):674-684

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Contributions by others to the thesis

The concept and design of the model utilised in this study was a joint collaboration between myself, John Fraser, Michael Reade, Elissa Milford, Sara Diab and Kimble Dunster. The specially designed instruments used to create crush injuries, contusions and fractures were constructed and calibrated by Kimble Dunster. Assistance with instrumentation of the animals during the experimental period was received from Sara Diab, Stephen Rayner and Kimble Dunster. All point of care viscoelastic testing was performed during the experimental period by Peter McGiffin. Chiara Palmeri assisted in the processing and evaluation of tissue histology. The ELISA assays for syndecan-1, activated protein C and thrombomodulin were performed with the assistance of John Cardinal. The hyaluranon ELISA assay was performed by Elissa Milford. The remaining serum and plasma assays were performed by Queensland Pathology. Data organisation was assisted by Elissa Milford, and statistical analysis was assisted by Marcella Kwan. Michael Reade and John Fraser reviewed drafts of this thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Trauma, acute traumatic coagulopathy, coagulation, haemorrhage, protein C, sheep, pre-clinical research

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ADP	adenosine diphosphate
AIS	abbreviated injury score
ALI	acute lung injury
aPC	activated protein C
aPTT	activated partial thromboplastin time
ATC	acute traumatic coagulopathy
ARDS	acute respiratory distress syndrome
Ca^{2+}	ionised calcium
CFT	clot formation time
CL	clot lysis
CT	clotting time
DAMPs	damage associated molecular patterns
DIC	disseminated intravascular coagulation
eTM	endothelial bound thrombomodulin
FFP	fresh frozen plasma
FV	factor V
FVIII	factor VIII
HMK	high molecular weight kininogen
IHC	immunohistochemistry
IL-1	interleukin-1
INR	international normalised ratio
ISS	injury severity score
ISTH	International Society on Thrombosis and Haemostasis
JAAM	Japanese Association for Acute Medicine
LY	lysis
MA	maximum amplitude
MCF	mean clot firmness
PAI-1	plasminogen activator inhibitor-1
PK	prekallikrein
PL	phospholipid
POC	point of care
PMP	platelet derived micro-particle
PT	prothrombin time

R	reaction time
ROTEM	rotational thromboelastometry
sTM	soluble thrombomodulin
SBL	Samm Border Leicester
TEG	thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIC	trauma induced coagulopathy
TNF-α	tumour necrosis factor-α
TM	thrombomodulin
tPA	tissue plasminogen activator
TXA	tranexamic acid
vWF	von Willebrand factor

CHAPTER 1: INTRODUCTION

Trauma remains a leading cause of death and disability worldwide, with approximately 10% of all deaths occurring due to traumatic injury (1). It predominately affects individuals younger than 44 years, with an average of 36 life years lost per trauma death (2, 3). However trauma has a wider impact than mortality statistics alone can illustrate. For every trauma fatality are two survivors who sustain serious or permanent disability, with over 1.8 billion disability adjusted life years lost annually as a result of traumatic injury (4). Trauma therefore has a significant financial impact on society as a consequence of healthcare costs and lost productivity, as well as having far reaching personal and emotional effects on the individual.

The causative mechanisms of trauma can be divided into blunt and penetrating injuries (5). Regardless of the initiating mechanism the temporal distribution and cause of mortality in trauma patients remains similar. Between one third and two thirds of trauma deaths occur prior to hospital admission and generally result from catastrophic head injury or major vessel disruption that is unresponsive to medical intervention and difficult to prevent (5-7). Of those trauma patients that survive until arrival to hospital the vast majority (up to 80%) will die, primarily during the first 48 hours of admission (3, 8). Despite advances in resuscitation, surgical management and critical care, uncontrollable haemorrhage remains the leading cause of preventable death during this period (3).

Haemostasis is precipitated by damage to the vascular endothelium, which triggers reflex local vasoconstriction and platelet activation in an attempt to minimise haemorrhage volume (9). Platelets adhere to the exposed sub-endothelial matrix via a collagen receptor and glycoprotein Ib which facilitates binding of von Willebrand factor (10). Adhesion triggers platelet activation resulting in changes in the cytoskeleton shape and the secretion of agonists such as adenosine diphosphate (ADP), thromboxane A2, adrenaline and serotonin to recruit additional platelets (10, 11). The rapid development of this platelet plug represents primary haemostasis and is effective in sealing small endothelial lesions in isolation. However platelet activation also triggers a cascade of pro-coagulant enzymes that results in the formation of a fibrin mesh to stabilise and support the platelet plug, a process known as secondary haemostasis.

The cascade model of coagulation depicts secondary haemostasis as two separate pathways triggered by different stimuli that ultimately result in the formation of fibrin: the contact activation (or intrinsic) pathway and the tissue factor (or extrinsic) pathway (figure 1) (12, 13). The cascade model accurately reflects the identity, function and interactions of the individual proteases involved in secondary haemostasis (12, 13). However it fails to take into account the contribution of the endothelium and other circulating cells to the coagulation process, and is therefore unable to fully explain many clinical bleeding syndromes. This has led to the proposal of the cell based model of haemostasis to better reflect the process of coagulation *in vivo* (14). The cell based model consists of 3 phases: initiation, amplification and propagation (figure 2). These phases are isolated to specific cell surfaces and suggest that the intrinsic and extrinsic pathways of coagulation are not redundant systems, but rather operate in parallel using different cell surfaces (14).

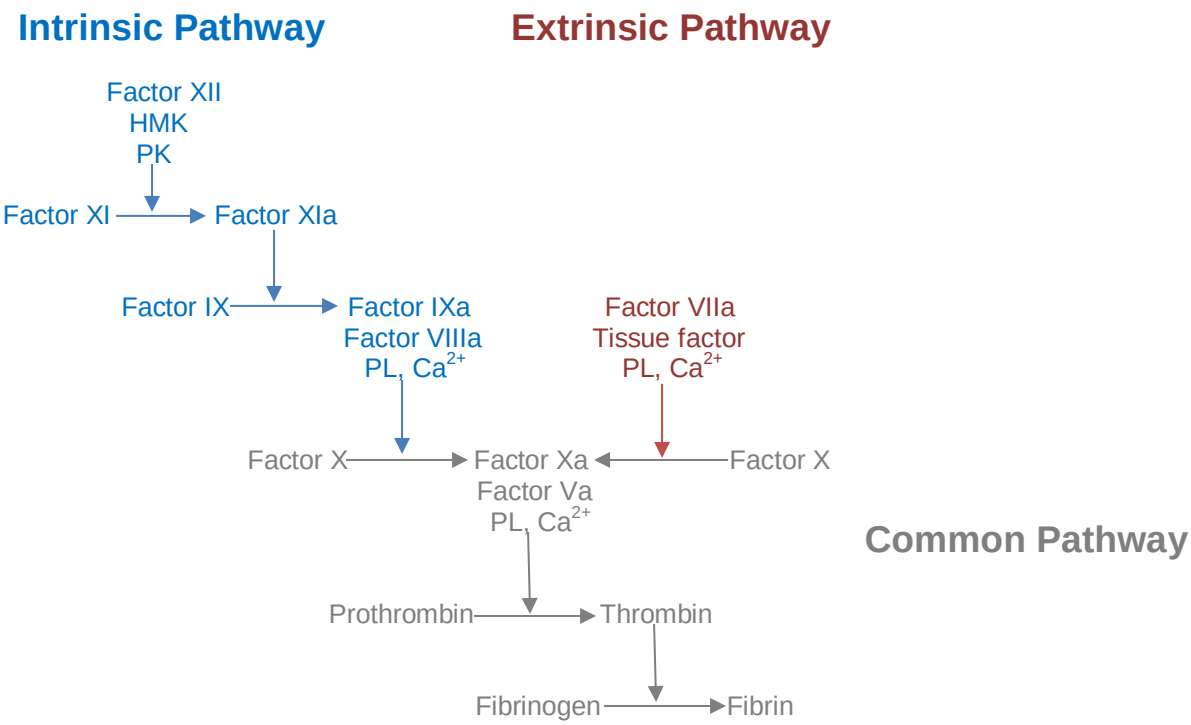

Figure 1. *The cascade model of coagulation. Adapted from Hoffman and Monroe 2001 (14)*

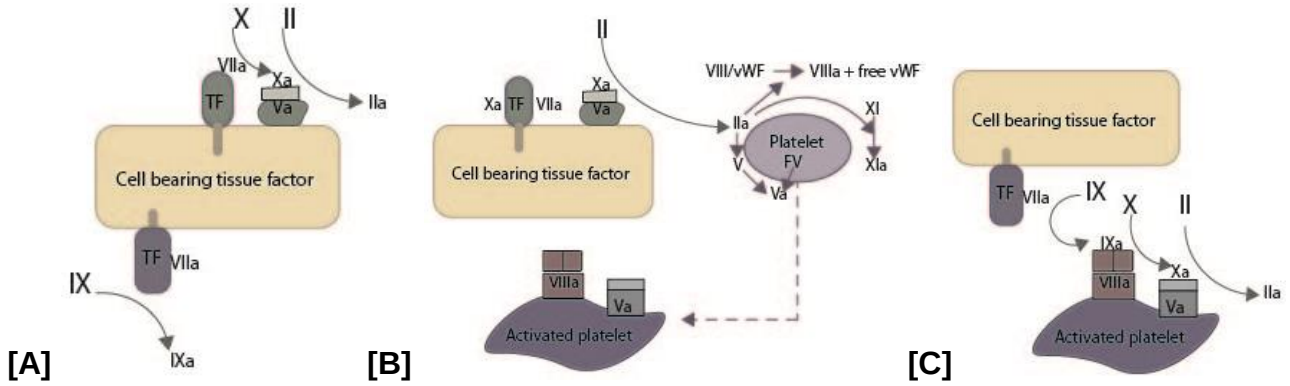

Figure 2. *The cell based model of coagulation. [A] Initiation phase, [B] Amplification phase, [C] Propagation phase. Adapted from Hoffman and Monroe 2001 (14)*

Normal pro-coagulant function is balanced by a number of endogenous anti-coagulant mechanisms that aim to restrict thrombin formation to the site of injury, avoid uncontrolled clotting of the entire vascular tree and inhibit local vessel thrombosis to maintain blood flow during periods of reduced perfusion (15-17). Intact endothelium prevents platelet adhesion to the thrombogenic subendothelial extracellular matrix and expresses heparin like molecules that activate anti-thrombins, inhibiting thrombin and several serine proteases including factors IXa, Xa, Xia and XIIa (15). The endothelium also expresses thrombomodulin (TM) which activates protein C and S to proteolytically inactivate factors Va and VIIIa (16), and produces tissue factor pathway inhibitor (TFPI) which prevents the ongoing tissue factor-factor VII interactions that initiate the extrinsic pathway (18).

Patients experiencing severe trauma often demonstrate impaired coagulation function, which complicates efforts to achieve effective haemostasis, increases transfusion requirements and contributes to higher mortality rates (19, 20). This trauma induced coagulopathy (TIC) has traditionally been attributed to the loss, dilution and dysfunction of coagulation proteases in response to fluid resuscitation, hypothermia and acidosis (20, 21). However it is now recognised that a mechanistically distinct acute traumatic coagulopathy (ATC) develops in the hyper-acute post trauma period independent of these factors (22, 23). The existence of this early coagulopathy has since been confirmed in a number of studies performed by independent research groups (22-26) with a strong association between coagulopathy and mortality reported in all studies (table 1).

Table 1. *The presence of an early endogenous coagulopathy has been confirmed by a number of independent research groups. In all studies the presence of coagulopathy was associated with a higher mortality rate.*

Authors	Number of patients	% with ATC	Mortality with ATC	Mortality without ATC
Brohi et al (22)	1,088	24.4%	46.0%	10.9%
MacLeod et al(23)	7,638	28.0%	19.3%	6.3%
Maegele et al(24)	8724	34.2%	28.0%	8.4%
Niles et al (25)	347	38.0%	24.0%	7.0%
Hess et al (26)	23,506	24.3%	26.4%	4.2%

The identification of ATC was originally based upon the traditional transfusion triggers recommended by massive transfusion guidelines (27, 28), namely a 50% prolongation of prothrombin time (PT), activated partial thromboplastin time (aPTT) or international normalised ratio (INR) (22, 23). However subsequent epidemiological work has shown a 20% increase in INR to be the clinically significant threshold for mortality and blood product requirements in trauma (29). More recently efforts have been made to characterise ATC using both thromoelastometery (ROTEM) or thromboelastography (TEG), reflecting the increasing use of viscoelastic point of care assays in the trauma setting (30-35). However there remains no universally accepted viscoelastic definition of ATC (36).

ATC appears to develop in response to a combination of tissue injury and systemic hypoperfusion (29, 37) and may simply represent the over-zealous activation of normal anti-coagulant processes. However the exact pathophysiological mechanisms underlying its development remain unclear. It has been suggested that activation of the protein C pathway may play a central role, as clinical studies demonstrate an association between protein C reduction, coagulopathy and mortality (37-39). The primary theory is that pathological activation of protein C results in systemic anticoagulation and hyperfibrinolysis via the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40). However the circulating concentration of PAI-1 is roughly ten times that of protein C, raising doubts about the ability of activated protein C (aPC) to deplete PAI-1 levels to the extent required to accelerate fibrinolysis (41). This has led to suggestions that ATC is a fibrinolytic form of disseminated intravascular coagulation (DIC) driven by increased tissue

plasminogen activator (tPA) release from the site of injury (21, 42-44). There is also emerging evidence to implicate platelet dysfunction and endothelial glycocalyx degradation in the development of ATC (45-48), although the exact contributions of these factors remains unclear.

Coagulation is also an integral component of the innate immune system, with coagulation dysfunction triggering a systemic inflammatory response that is further compounded by immunologic responses to blood product transfusion and fluid resuscitation (49, 50). Haemostatic resuscitation using combinations of red blood cells, plasma, platelets, cryoprecipitate and fibrinogen remains the core therapeutic approach to severe trauma (51, 52), and has been combined with adjuncts such as prothrombin complex, recombinant activated factor VII and tranexamic acid (TXA) to further improve haemorrhage control (53).This may improve short term mortality rates (52); however the effect on the subsequent pro-coagulant state still needs to be quantified. TXA has also been shown to impede neutrophil adhesion pathways via the inhibition of plasmin (54, 55). This may further alter the immune response in a patient population susceptible to sepsis and influence the medium-long term complication rate. Targeted treatment strategies are required to further improve outcomes for patients with ATC; however the development of these is currently restricted by the limited mechanistic understanding of the condition.

Pre-clinical animal research has become an attractive option for investigating the pathogenesis and management of ATC. Human research in the trauma setting can be problematic, as the dynamic nature of traumatic injury creates difficulties in the recruitment of patients to randomized controlled trials and increases the risk of bias and confounders in both prospective and retrospective studies (56, 57). Animal models provide an alternative option as they facilitate the controlled and systematic evaluation of isolated insults *in vivo*, which may enhance mechanistic understanding and identify novel therapeutic targets. However animals are genetically distinct from humans, and demonstrate differences in coagulation assay parameters, coagulation factor concentrations and platelet function that may alter the susceptibility to ATC (58-62). For an animal model to be clinically relevant there needs to be more similarities than differences between the human coagulation system and that of the chosen animal species.

There are a number of published animal models that have attempted to characterise changes in coagulation function in response to trauma and haemorrhage (63, 64).

However there remains no consensus as to the species or type of traumatic insult that most closely replicates the human condition. Rodents have consistently demonstrated the ability to achieve clinical definitions of ATC in response to trauma and haemorrhage (29, 40, 65), however they are limited by animal size and recognized differences in coagulation function (58). Pigs are the only large animal species that has been used for evaluating ATC, which would appear appropriate given the established physiological similarities between pigs and humans (66). However comparative studies of coagulation function have shown pigs to be relatively hypercoagulable compared to humans, with protein C levels only 36% of human values (58, 60). Given the hypothesized role of protein C in the development of ATC it is possible that this fundamental difference may impede the ability to successfully model ATC in pigs. The only published porcine model to develop coagulation changes consistent with clinical definitions of ATC reports a 25% mortality rate in the trauma group (67). This may reflect the severity of injury required to produce ATC in this species and raises concerns over the ethical acceptability of this model. The predisposition for porcine models may therefore be limiting information about ATC that could be obtained from alternative large animal species.

Sheep have been widely used in biomedical research to model a number of human pathologies including asthma (68, 69), osteoporosis (70), sepsis (71) and acute lung injury (ALI) (72-74), as they also share many physiological similarities with humans (75-77). Comparative studies of human, porcine, rodent, canine and ovine coagulation function using routine coagulation tests, rotational thromboelastometry (ROTEM) and clotting factor assays also suggest that the human coagulation system demonstrates the greatest similarity with that of sheep (58, 59). However there are no current published ovine models of ATC. The development of an ovine model of ATC may therefore improve understanding of pathophysiology and better inform subsequent human studies.

The primary aims of this thesis were three fold. The first was to undertake a systematic review of the literature to identify and evaluate existing animal models of ATC, to better inform the design of an alternative large animal model. The second was to develop an ovine model of trauma and haemorrhage that demonstrated coagulation changes consistent with ATC as defined by INR, aPTT and ROTEM. The third was to evaluate the relationships between coagulopathy, the protein C pathway, endothelial glycocalyx, platelet function and fibrinolysis within the ovine model to better inform future mechanistic studies.

CHAPTER 2: AN OVERVIEW OF ACUTE TRAUMATIC COAGULOPATHY

2.1 Background of Acute Traumatic Coagulopathy

Coagulopathy in trauma has classically been considered an iatrogenic process developing late after injury in response to haemodilution, acidosis and hypothermia (20). In 2003 two separate retrospective clinical studies described an endogenous coagulopathy present at hospital admission that had developed in the absence of these physiological derangements (22, 23). The existence of this acute traumatic coagulopathy (ATC) has since been confirmed in multiple publications from independent research groups and has changed our comprehension of coagulopathy in trauma (24-26, 29, 30, 32, 39). It is now understood that haemostatic equilibrium is disrupted early post trauma by an endogenous dysfunction which is further aggravated by medical interventions promoting the development of haemodilution, acidosis and hypothermia (figure 3).

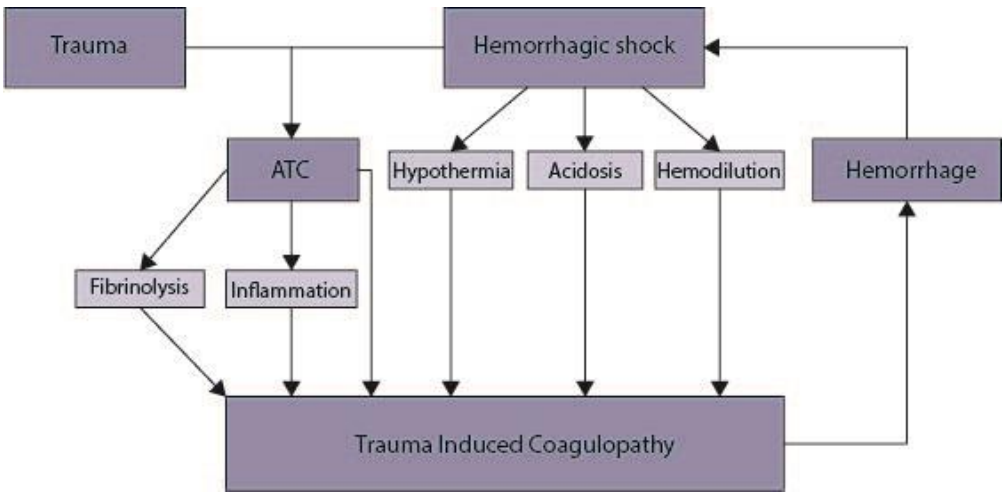

Figure 3. *ATC is an endogenous coagulation dysfunction developing in response to tissue injury and haemorrhagic shock. Acidosis, hypothermia and haemodilution simply exacerbate this underlying dysfunction, signalling progression to TIC.*

The reported incidence of ATC at hospital admission varies from 24-41% depending on the diagnostic criteria that is used (table 2) (22-25, 29, 32, 39). However in all studies it is associated with a negative impact on patient outcomes. ATC has been independently associated with higher transfusion requirements, increased incidence of post injury multi-organ failure, longer critical care unit stays and a four-fold increase in the risk of mortality (22-25, 29) .The inability to control bleeding is the biggest factor contributing to mortality

and morbidity in patients with ATC (78). However the presence of ATC is also a strong predictor of venous thromboembolism which can further complicate the clinical course (79) and knowing when to instigate thromboprophylaxis is difficult in patients presenting with coagulopathy.

Table 2. *Various definitions of ATC have been proposed in the literature. ATC is associated with a negative impact on patient outcomes regardless of the definition used.*

Authors	Number	Definition	% with ATC	Mortality with ATC	Mortality without ATC
Brohi et al (22)	1,088	PT>18s or aPTT>60s or INR>1.5	24.4%	46.0%	10.9%
MacLeod et al(23)	7,638	PT>14s or aPTT>34s	28.0%	19.3%	6.3%
Maegele et al(24)	8724	PT <70% or platelets $<100x10^9$	34.2%	28.0%	8.4%
Niles et al (25)	347	INR>1.5	38.0%	24.0%	7.0%
Frith et al (29)	3,646	INR>1.2	36.2%	22.7%	7.0%
Davenport et al (32)	300	ROTEM A5≤35mm	18.0%	23.4%	6.2%
Cohen et al (39)	1,245	INR>1.3 or aPTT>35s	41.6% by INR 20.5% by aPTT	32.6%	12.5%

PT = prothrombin time, aPTT = activated partial thromboplastin time, INR = international normalised ratio, ROTEM A5 = clot amplitude at 5 minutes using rotational thromboelastometry.

The recognition of ATC has driven changes in the clinical management of traumatic injury. Resuscitation practices in trauma have shifted away from crystalloid based regimens towards the use of whole blood, or red cell concentrates in combination with plasma, fibrinogen, cryoprecipitate and platelets (51, 52, 80, 81). Improvements in survival rates have been associated with these ‘damage control’ or ‘haemostatic’ resuscitation regimens that have been retrospectively attributed to improvements in haemostatic function (51, 52, 82). However a recent study by Kahn and colleagues suggests this is not the case, with ongoing deterioration of coagulation function evident in the face of haemostatic resuscitation (80). Rather than directly addressing coagulopathy it is possible that factors such as the prevention of haemodilution, type of injury, rate of haemorrhage, survivor bias or other confounders may have contributed to the survival benefits associated with

haemostatic resuscitation regimens (83). The optimum composition of blood products for resuscitation is also a point of contention, with the ratios and types of products used varying between countries and institutions (84-86). This divergence in clinical practice reflects a lack of knowledge regarding the benefits and risks of individual blood components in the management of ATC.

2.2 The pathophysiology of Acute Traumatic Coagulopathy

A combination of tissue injury and tissue hypoperfusion has been shown to be a necessary pre-requisite for the development of ATC in clinical studies (25, 29, 37, 39, 87-90), *in vitro* experiments (91) and animal models (29, 40, 65). A normal base deficit is not associated with coagulopathy in trauma regardless of the injury severity (29, 37, 92, 93). However systemic hypoperfusion has a dose-dependent effect on coagulation function in the face of tissue injury (39). ATC is most likely to occur when a base deficit of more than 6mmol/L is combined with an injury severity score (ISS) greater than 15, and the incidence and severity of coagulopathy in patients with haemorrhagic shock increases significantly when the ISS is 25 or greater (29).

The presence of ATC is characterised by systemic hypocoagulability, dysfibrinogenaemia and hyperfibrinolysis (89, 94). However the underlying mechanisms triggering the development of ATC remain unclear and this current knowledge gap remains a key barrier to the development of targeted treatment strategies for this subset of patients. Protein C activation, tissue factor release, endothelial glycocalyx shedding, platelet dysfunction and fibrinolysis have all been hypothesised to play a role in the development. However the evidence for many of these is limited and their role in the haemostatic response to tissue injury and shock requires further clarification.

2.2.1 Activation of the protein C pathway.

Activation of the protein C pathway is considered by many to be instrumental to the development of ATC, with the accompanying protein C depletion postulated to contribute to the increased risk of post injury multi-organ failure and mortality (37, 38). Clinical studies have demonstrated a correlation between protein C reduction or activated protein C (aPC) increase, prolonged PT/aPTT and reduced clot strength (37-39, 95). Mechanistic

evaluation in a murine model of ATC has added further weight to the theory, with coagulopathy prevented by the administration of aPC antibodies (40).

Protein C is a vitamin K dependent glycoprotein that is activated on the surface of endothelial cells by thrombin bound concurrently to the endothelial protein C receptor (EPCR) and the transmembrane glycoprotein thrombomodulin (TM) (16, 37). It is postulated that upregulation of TM expression occurs in response to tissue hypoperfusion (37, 39). When this is combined with increased thrombin generation from tissue trauma an increase in thrombin-TM complex formation occurs, resulting in pathological protein C activation (figure 4)(16). aPC then inhibits the coagulation cascade via the inactivation of factors Va and VIIIa and promotes fibrinolysis through the consumption of PAI-1 (figure 5)(16, 38, 96, 97). The protein C hypothesis therefore provides an explanation for both systemic anticoagulation and hyperfibrinolysis, although the magnitude of these effects in the development of ATC has not been quantified.

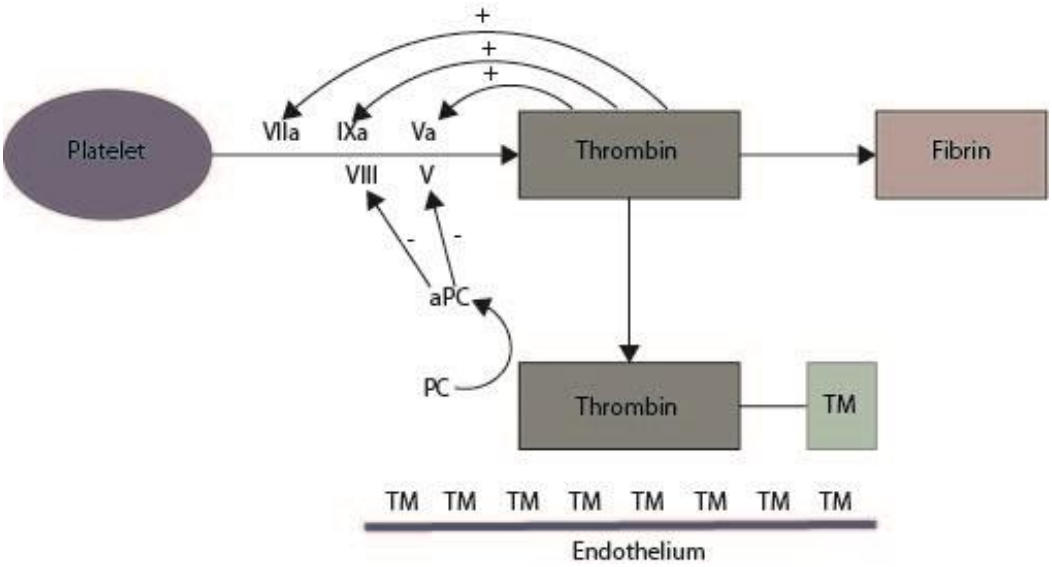

Figure 4. *Thrombomodulin is released from activated endothelial cells and combines with thrombin to activate protein C. Activated protein C then inhibits coagulation by inactivating factors Va and VIIIa. Adapted from Brohi et al 2007 (98)*

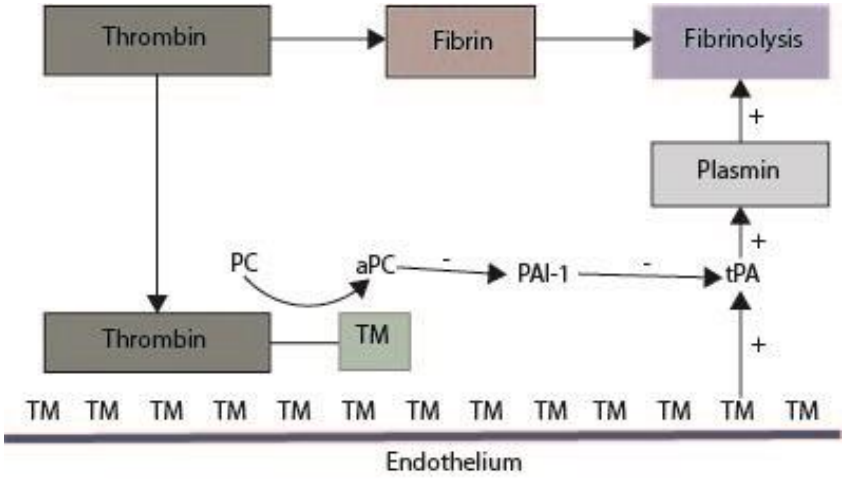

Figure 5. *Activated protein C inhibits plasminogen activator inhibitor-1, increasing the production of plasmin and promoting fibrinolysis. Adapted from Brohi et al 2007 (98)*

The significance of the protein C pathway in ATC has recently been questioned by Campbell and colleagues (44) who have shown that platelet and plasma factor Va pools are resistant to cleavage by aPC *in vitro,* both at concentrations observed in trauma patients and following the therapeutic doses of recombinant human aPC used in sepsis. In addition they found no evidence of fibrinolysis, arguing that the high circulating levels of PAI-1 are unlikely to be inactivated to the extent required to drive the fibrinolytic picture seen with ATC. The application and interpretation of these findings from a static system is limited given the complex, dynamic process of coagulation *in vivo*. Further evaluation of the interaction between the protein C pathway, platelet function and fibrinolysis is warranted to better understand their contribution to ATC.

2.2.2 The importance of fibrinogen and fibrinolysis

Haemostasis relies upon fibrinogen for adequate clot formation (99). Hypofibrinogenemia is well documented in clinical and experimental studies of ATC, and has been correlated with both early and late mortality (100-103). Early fibrinogen replacement has been shown to improve outcomes and correct coagulopathy (87, 100, 104, 105). However there is controversy regarding the critical level for replacement. Levels below 2.29g/L were associated with increased morbidity and mortality rates in a large multicentre observational study (94), which is well above the threshold for replacement of 1.0 g/dL recommended by current guidelines (106). This incongruity reflects a lack of understanding of the underlying benefits and risks of fibrinogen replacement in trauma. A randomised controlled trial to better evaluate the role of fibrinogen replacement in trauma is in the preliminary stages

(107). In the interim a controlled animal model of ATC may facilitate mechanistic evaluation of fibrinogen replacement and improve understanding of the role of fibrinogen replacement in trauma.

The cause of hypofibrinogenaemia in ATC is unclear. Acidosis, hypothermia and hemodilution have all been shown to lower fibrinogen levels (108), however these are not significant contributors to ATC. The formation of weak clots that are susceptible to increased fibrinolysis or fibrinogenolysis has been proposed as a cause (109). It is also possible that activation of the protein C pathway and subsequent disinhibition of the fibrinolytic pathways may lead to fibrinogen consumption and subsequent hypofibrinogenaemia (110).

Fibrinolysis appears to develop in a subset of patients with ATC (39, 96, 111, 112) and is strongly correlated with poor clinical outcomes (96, 111-115). The CRASH-2 study provides level I evidence of the importance of fibrinolysis, demonstrating a significant survival benefit following administration of the anti-fibrinolytic tranexamic acid (TXA)(116, 117). However a cautious approach to the empirical use of TXA is required. As ATC progresses a pro-thrombotic state begins to predominate (118), with a high incidence of thromboembolic complications evident following major trauma (79, 119). The development of this pro-coagulant tendency has been attributed to inflammation, circulating microparticles and dysregulation of tissue factor and thrombin, which act to increase PAI-1 production and shutdown fibrinolysis (120, 121). The survival benefits from TXA in the CRASH-2 study were time limited, with increased mortality rates observed if administered more than 3 hours following injury (116). Administration of TXA after the benefit of reduced fibrinolysis has passed may potentiate the prothrombotic state, increasing the risk of thromboembolic complications. TXA has also been shown to impede the adherence and transmigration of neutrophils which may have an indirect effect on outcomes (54). Impeding leucocyte-endothelial interactions has been shown to dampen the systemic inflammatory response and improve outcomes in animal models of sepsis (122, 123). Therefore TXA administration could theoretically play a role in decreasing the risk of sepsis in trauma, although stage 2 and 3 human trials have shown minimal benefit from these anti-adhesion therapies (123, 124). Conversely the late administration of TXA could theoretically contribute to an increased risk of sepsis and mortality by inhibiting the innate immune response, predisposing the patient to systemic bacterial infection (122).

The mechanism behind fibrinolysis in ATC is unclear. Increased levels of tissue plasminogen activator (tPA) are evident in trauma, and PAI-1 is the primary inhibitor of tPA (96). As previously mentioned it has been suggested that PAI-1 consumption by aPC might disinhibit fibrinolysis, contributing to the fibrinolytic picture observed (96, 97). Others argue the increased tPA levels are due to massive release following injury, which when augmented by increased thrombin production, catecholamine and vasopressin release triggers fibrinolysis (44, 125). Recent work in a rodent model has demonstrated that TEG detectable fibrinolysis is inhibited by tissue injury and promoted by haemorrhagic shock, although the hypofibrinolytic phenotype of rats makes the significance of this finding unclear (126). At face value it may also appear that ATC is actually DIC with a fibrinolytic phenotype, as the initial changes of ATC are positive on both International Society on Thrombosis and Hemostasis (ISTH) and Japanese Association for Acute Medicine (JAAM) scoring systems (125, 127, 128). However there is no histopathological evidence of inappropriate disseminated clot formation in trauma patients to suggest ATC and DIC are one and the same (129).

2.2.3 Endothelial injury

The endothelial glycocalyx is a negatively charged anti-adhesive and anti-coagulant surface layer that protects the endothelial cells and maintains vascular barrier function (130). Traumatic injury and shock result in tissue ischaemia, activation of the inflammatory system and stimulation of the neuro-humoral axis with a subsequent catecholamine surge (45, 131-134). This can lead to endothelial cell activation, glycocalyx degradation and luminal expression of anticoagulant/profibrinolytic proteins (45, 135) with emerging evidence suggesting these factors may play a role in ATC (45, 46, 132). Trauma patients have demonstrated an increase in syndecan-1, a soluble marker of glycocalyx shedding, which correlates with the severity of shock, levels of circulating catecholamines, tissue injury and markers of fibrinolysis (45). Elevated syndecan-1 is also associated with a higher incidence of ATC and mortality regardless of ISS, suggesting the downstream effects of trauma modulate the response rather than the injury itself (45).

Endothelial glycocalyx shedding appears to trigger thrombin generation and fibrinolysis, which in the presence of increased soluble thrombomodulin (sTM) from damaged endothelial cells may enhance protein C activation (45, 135). A volume of plasma containing heparin like substances is also held within the glycocalyx (136), and release of this following degradation may lead to direct anticoagulant effects from endogenous

heparinisation (46). Electron microscopy data from experimental models of haemorrhagic shock suggests that the endothelium may be a potential therapeutic target in ATC (137). However further work to better evaluate the microvasculature and its role in ATC is still required.

2.2.4 Role of platelet function and microparticles

The cell based model of haemostasis recognizes the fundamental role of platelets in the balanced assembly of a fibrin clot (14), with a recent study demonstrating that platelets contribute to two thirds of overall clot strength (99). Minor reductions in the admission platelet count of trauma patients are predictive of mortality, even if they remain within the normal range (138, 139). Decreased responsiveness to collagen, ADP and arachidonic acid agonists has also been demonstrated in trauma using TEG and whole blood aggregometry (Multiplate), and is strongly associated with mortality (47, 48, 140). These observations may explain the improved outcomes associated with early platelet transfusion in trauma, with the degree of improvement further impacted by the quality of transfused platelets (85, 141, 142).

The role of platelet dysfunction in ATC remains obscure. It has been suggested that initial platelet hyperactivation in trauma may render platelets unresponsive to subsequent stimulation, resulting in reduced aggregation parameters and decreased clot strength (143). A similar phenomenon has been observed in conditions such as transplant rejection and thrombotic thrombocytopaenic purpura, in which acquired defects in platelet function develop following prolonged activation *in vivo* (144). Platelet dysfunction may also play a role in hyperfibrinolysis. ADP release into the circulation has been shown to increase sensitivity to tPA, resulting in increased fibrinolytic activity (145). However mechanistic studies evaluating platelet function in trauma are limited and further work is required to better characterise the contribution to ATC.

Recent work has also focused on the contribution of microparticles to coagulopathy. These small vesicles are derived from blood and endothelial cells and contribute to normal haemostatic function (146). Platelet derived microparticles (PMPs) are the most abundant and are highly pro-coagulant (147, 148). The PROMMT study demonstrated an association between decreased levels of PMPs and poor clot strength, increased blood product requirements and mortality in coagulopathic trauma patients (149). An alternate study demonstrated no significant difference in microparticle concentration with time in

trauma patients (150), while a third study reported elevated PMPs in trauma patients at hospital admission that were implicated in the development of post injury multi-organ failure (151). Alterations in PMP levels may be a separate component of platelet dysfunction developing independently to impaired aggregation capacity, and may play an important part in the haemostatic and inflammatory responses to trauma. However further work is required to determine the precise contribution to ATC.

2.3 Current definitions of Acute Traumatic Coagulopathy

Laboratory diagnosis is required for identification of ATC; however there remains no consensus regarding the laboratory definition that should be used. ATC was originally characterised by Brohi et al as PT > 18s, aPTT > 60s or INR >1.5 and by MacLeod et al as a PT >14s and aPTT > 34s (table 2)(22, 23). Subsequent epidemiological work has found a 20% increase in INR to be the clinically significant threshold for blood product requirements and mortality, with the previously cited thresholds failing to identify 16% of trauma patients experiencing poorer outcomes (29). These traditional assays of coagulation function remain the basis for identification of ATC as they are readily available and have demonstrated an ability to predict mortality and need for transfusion in the trauma patient (25).

In recent times the value of these traditional assays in the contemporary management of ATC has been questioned. While prolongation of these assays has been associated with poorer patient outcomes it is conceivable that they may simply be a marker of injury severity, rather than an accurate reflection of coagulation function. These tests were initially developed to identify specific coagulation factor deficiencies and have been shown to be poor predictors of haemorrhage in the face of multiple acquired deficiencies (152, 153). The assays are performed on platelet poor plasma and reflect only the initial 20-60 seconds of clot formation, failing to take into account the contribution of platelets to haemostasis, the role of fibrinolysis or thrombin generation, or the global interaction between coagulation enzymes (32). Furthermore the turn-around times of 30-60 minutes often negates the value of results in the rapidly evolving setting of acute trauma (32). This prolonged turnaround time could be addressed through the use of point of care (POC) INR devices, which have shown to correlate well with formal laboratory testing in the trauma setting (154-157). However the acknowledged limitations of these traditional assays

remain, which has precluded widespread uptake of POC INR testing in the trauma setting (158).

Viscoelastic coagulation testing is widely used in cardiac and transplant surgery to guide resuscitation, and has been associated with decreased blood product use and improved outcomes (159-163). These tests are performed on whole blood and produce a trace that is representative of [1] platelet activation, [2] thrombin burst, [3] function of plasma proteins including fibrinogen and [4] the fibrinolytic system (164) (figure 6). Unlike the traditional assays of coagulation function viscoelastic tests provide an early and more comprehensive assessment of secondary haemostasis (164, 165). The need for an accurate and rapidly available diagnostic test of ATC has prompted the increased utilisation of viscoelastic testing in the trauma setting.

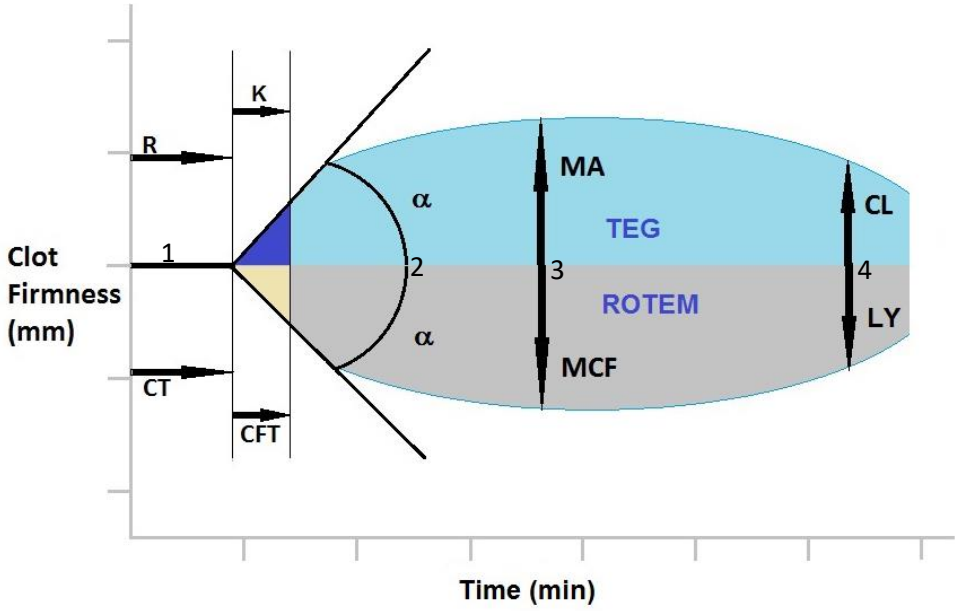

Figure 6. *Schematic viscoelastic trace demonstrating the commonly reported variables of TEG (upper part) and ROTEM (lower part).Reaction time (R)/clotting time (CT) is partially representative of platelet function (1), alpha angle (α) reflects the thrombin burst (2), maximum amplitude (MA)/maximum clot firmness (MCF) reflects the function of plasma proteins (3) and clot lysis (CL/LY) represents fibrinolysis (4).*

Attempts to define ATC using both rotational thromboelastometry (ROTEM) and thromboelastography (TEG) have been made (30-33, 166-170). ATC has a viscoelastic profile characterised by slow clot formation time and reduced clot amplitude (32, 33, 167-169). ROTEM EXTEM A5 values ≤35mm have been associated with increased transfusion

requirements and mortality compared to an INR > 1.2 (32, 34). Others suggest an EXTEM A10 ≤40mm correlates best with platelet count, fibrinogen level and blood product requirements (30, 170). Similar findings are evident with TEG, with maximum amplitude (MA) <55mm, K-time >2.5s and R value >1.1s associated with poor outcomes and increased need for transfusion (33, 167, 168). However there remains no universally accepted viscoelastic definition of ATC (36, 93).

The ability of viscoelastic testing to distinguish between different haemostatic abnormalities suggests that it may provide a means of individualizing haemostatic resuscitation in the trauma patient (33, 113). However there are no viscoelastic based resuscitation algorithms for trauma that have been validated by randomised trials. Viscoelastic tests also fail to take into account the contribution of the endothelium to coagulation, which may play an important role in platelet and fibrinogen function (95). These assays are also relatively insensitive to the detection of fibrinolysis. Over 80% of trauma patients with an ISS > 15 demonstrate fibrinolysis using plasmin-antiplasmin assays (112), however only 5-10% of these patients are detected using viscoelastic testing (96, 111, 112). Further work is required to identify the viscoelastic triggers that will improve outcomes for patients with ATC. The development of additional tests to fully evaluate coagulation function may be required to facilitate individualised treatment strategies for the trauma population.

CHAPTER 3: EXPERIMENTAL ANIMAL MODELS OF TRAUMATIC COAGULOPATHY: A SYSTEMATIC REVIEW

van Zyl N, Reade MC, Fraser JF. *Experimental animal models of traumatic coagulopathy: A systematic review.* Shock 2015;44(1):16-24

3.1 An introduction to this systematic review

Pre-clinical animal research is an attractive option for evaluating the pathophysiology and management of ATC. The variable and dynamic nature of trauma and its management means that human trauma research can be limited by confounders, difficulties with patient recruitment and bias (56, 57). Animal models offer an alternative as they allow the systematic evaluation of isolated insults *in vivo*. This may reduce the impact of confounders whilst simultaneously improving mechanistic understanding. Based upon the perception of an iatrogenic process a number of animal models have investigated the effects of hypothermia, acidosis and haemodilution on systemic coagulation function (63, 64). However to study ATC effectively animal models need to reflect our current understanding of the condition as an endogenous response to tissue injury and hypoperfusion.

The use of animals for medical research has significant ethical considerations. All proposed animal experimentation is now appropriately scrutinised by institutional animal ethics committees and must encompass methods to protect and promote the welfare of animals (171). These methods include means to replace animals with alternatives where possible, reduce the number of animals used to the minimum required for statistical validity and refine experimental techniques to reduce the overall impact on the animals (171). Consideration of these ‘3R’ principles is therefore essential in the development of any animal study in order to maximise benefits and improve ethical acceptability.

This paper addresses the first aim of this thesis: to perform a systematic review of the literature in order to better inform the design of the animal model utilised in this thesis. Existing animal models of traumatic coagulopathy published following the recognition of ATC in 2003 were identified. The initiating mechanism of coagulopathy, mechanism of

injury, type of haemorrhage and animal species used were then assessed for clinical relevance, variability of response and survivability of injury. Collating this information identified experimental techniques that could be refined and combined in different ways in future animal models. It also identified data upon which to base sample size and power calculations, facilitating the development of a novel ovine model in an ethically acceptable manner

3.2 Reprint of accepted manuscript.

Experimental animal models of traumatic coagulopathy: a systematic review.

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Abstract

Introduction

Perturbations in coagulation function are common following trauma and are associated with poor clinical outcomes. Traditionally considered an iatrogenic process, it is now recognized that an acute coagulation dysfunction develops prior to medical intervention. The mechanisms underlying the development of this acute traumatic coagulopathy (ATC) remain poorly understood. Pre-clinical animal research is a necessary adjunct to improve mechanistic understanding and management of this condition. This review aims to identify and evaluate existing animal models of traumatic coagulopathy for clinical relevance.

Methods

A structured search of MEDLINE/Pubmed was performed in September 2014 in accordance with the PRISMA guidelines.

Results

A total of 62 relevant publications describing 27 distinct models of traumatic coagulopathy were identified. Porcine models predominated and hemodilution in isolation or in combination with hypothermia and/or acidosis was the principal mechanism for inducing

coagulopathy. Acute coagulation changes in response to tissue injury and hemorrhage were evident in 5 publications, and pathophysiological evaluation of postulated mechanisms was performed in 3 studies.

Conclusions

There are few clinically relevant animal models that reflect the contemporary understanding of traumatic coagulopathy. This relative deficiency highlights the need for further development of valid and reproducible animal models of trauma. Well-designed models will facilitate improved mechanistic understanding and development of targeted treatment strategies for traumatic coagulopathy.

Keywords: trauma, coagulation, hemorrhage, clotting function, pre-clinical research

Introduction

Trauma remains the principal cause of death and disability in people aged between 1-44 years (1). Severe hemorrhage is the leading cause of preventable death in the trauma population, responsible for up to 40% of all deaths occurring within 24 hours of trauma (78, 172).

Coagulation dysfunction following trauma is well recognized and influences the ability to achieve effective hemostasis. Trauma induced coagulopathy (TIC) was traditionally attributed to the loss, dilution and dysfunction of coagulation proteases in response to fluid resuscitation, hypothermia and acidosis (20). However, over the past decade derangements in coagulation function developing independent of these factors have been identified in the hyper-acute post-trauma period (22, 23). Identification of this acute traumatic coagulopathy (ATC) has altered the perceived natural history of TIC. The physiologic derangements that characterize TIC are now recognized to develop in a hemostatic system that is already unbalanced by an endogenous coagulation dysfunction.

The reported incidence of ATC in trauma patients at hospital admission varies between 24-41%, reflective of the multiple definitions of ATC that have been proposed (22, 23, 29, 39). ATC was originally characterized as a 50% prolongation in prothrombin time (PT), activated partial thromboplastin time (APTT) or international normalized ratio (INR) (22, 23). More recently an INR >1.2 has been shown to be the clinically significant threshold for increased mortality and blood product requirements (29). The identification of ATC at

hospital admission continues to be based upon these traditional assays of coagulation function. However these assays have limitations in the contemporary management of traumatic coagulopathy as they are performed on platelet poor plasma, reflect only the initial 20-60 seconds of clot formation and usually require 30-60 minutes for processing (32). Efforts have been made to define ATC with point-of-care viscoelastic coagulation measurements using both thromboelastometry (ROTEM) or thromboelastrography (TEG), however there is no current universally accepted viscoelastic definition, assay or validated algorithm for ATC (30-32, 95).

The pathophysiological mechanisms underlying the development of ATC remain poorly understood. A combination of tissue injury and shock resulting in tissue hypoperfusion appears to be a necessary requirement, as neither of these insults in isolation is associated with deranged coagulation function (29, 37, 39). Activation of protein C (aPC) may play a central role by diverting hemostasis towards hypocoagulability and hyperfibrinolysis through the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40, 173). Dissenters of this theory suggest that the concentrations of aPC associated with ATC are insufficient to produce these effects (44). It has been postulated that ATC is disseminated intravascular coagulation (DIC) with a fibrinolytic phenotype induced by tissue factor release from the site of injury, although histologic evidence of DIC is absent (43, 129). There is also emerging evidence to suggest that platelet dysfunction and endothelial glycocalyx degradation make a significant contribution to ATC, with catecholamine induced endothelial damage proposed as an alternative initiating mechanism for coagulopathy (44-47, 132).

The adverse outcomes related to ATC are not limited simply to the effects of acute blood loss. Coagulation dysfunction can trigger a de novo systemic inflammatory response which is compounded by immunologic responses to blood product transfusion and fluid resuscitation (49, 50). This results in an increased risk of post injury multi-organ failure, sepsis and thromboembolic complications in the long term (98). The current core therapeutic approach to patients with traumatic injury is the use of hemostatic resuscitation to restore circulatory homeostasis and control hemorrhage (51, 81). Whilst this improves mortality rates it may influence the development of a subsequent pro-coagulant state (121). Further improvements in patient outcomes require the development of targeted treatment strategies for this population, along with an understanding of when to switch from pro-coagulant to anti-coagulant therapies. This is currently restricted by our limited

knowledge of the pathogenesis of ATC and underlines the need for improved mechanistic understanding.

The pathogenesis and management of ATC has become a prime target for pre-clinical research, as the nature of traumatic injury creates difficulties for human research in the emergency setting. Recruitment to randomized controlled trials is difficult in a population where treatment begins in the field and consent can be difficult to obtain, reducing study power and increasing the risk of bias (56). Retrospective human studies can have multiple confounders, making it difficult to separate association and causation in the acutely ill trauma patient. They are also reliant on the quality of available data and are associated with survivor bias (174).

Animal models offer the opportunity to investigate isolated insults *in vivo* in a controlled and systematic fashion. This can improve mechanistic understanding of pathophysiology, facilitate the development of novel therapeutic strategies and provide a platform for the evaluation of existing and developing therapeutic interventions. While there has been considerable interest in developing animal models of ATC, coagulation mechanisms vary between species (58-60). For these models to be clinically relevant there needs to be more similarities than differences between the human coagulation system and that of the model investigated. As yet there appears no consensus as to the species, traumatic insult and resuscitation strategies that most closely replicate the human condition.

The aim of this review was to identify the experimental animal models that have been used to investigate traumatic coagulopathy following the identification of ATC in 2003. The models were evaluated for clinical relevance, ability to characterize underlying pathophysiologic mechanisms and ability to evaluate the effectiveness of hemostatic interventions. A systematic review of the literature was performed.

Methods

Search Strategy

The indexed online database MEDLINE/Pubmed was searched in September 2014 using the terms ("animals"[MeSH Terms] OR animal[All Fields]) OR preclinical[All Fields] OR model[All Fields] AND ("injuries"[Subheading] OR "injuries"[All Fields] OR "trauma"[All Fields] OR "wounds and injuries"[MeSH Terms] OR ("wounds"[All Fields] AND "injuries"[All Fields]) OR "wounds and injuries"[All Fields]) AND ("haemorrhage"[All Fields] OR

"hemorrhage"[MeSH Terms] OR "hemorrhage"[All Fields]) AND ("blood coagulation disorders"[MeSH Terms] OR ("blood"[All Fields] AND "coagulation"[All Fields] AND "disorders"[All Fields]) OR "blood coagulation disorders"[All Fields] OR "coagulopathy"[All Fields]). The search was limited to studies published in English within the past 10 years to encompass the time period following identification of ATC.

Selection criteria

Abstracts and citations identified by the search were screened for relevance. Full publications of studies considered relevant were retrieved and reviewed. Additional relevant publications cited within the retrieved articles were also reviewed. Publications were included if they described an animal model of trauma or hemorrhage and reported systemic measures of coagulation function. Animal models of burn injury and traumatic brain injury were excluded due to acknowledged differences in the processes leading to coagulopathy in these conditions (175, 176).

Results

The search process produced 448 abstracts, with an additional 7 relevant publications identified from other sources. A total of 71 publications were considered relevant and their full text reviewed. Following exclusions a total of 62 publications were available for review (see Figure 1.) Of these 62 publications 26 were conducted to characterize the time course of coagulopathy or investigate pathophysiological mechanisms associated with the development of coagulopathy. The remaining 36 studies aimed to investigate a therapeutic intervention. Many of the studies were published by the same authors or research groups and described previously published models with only minor variations in study protocol. The studies that were considered to represent an original and distinct model of traumatic coagulopathy are summarized in table 1.

Study Characteristics

Six different animal species were featured in the studies evaluated (see table 1). Porcine models predominated (n=45), with the remainder comprised of rat (n=10), rabbit (n=4), sheep (n=1), mouse (n=1) and hamster (n=1) models. All studies utilized general anesthesia administered via the intravenous, intraperitoneal and/or inhalational routes.

Coagulation function was assessed by traditional plasma based assays (PT, APTT, INR, ACT) and/or viscoelastic assays (TEG or ROTEM). In most studies tests of coagulation

function were used to establish the presence/absence of coagulopathy following the initiating mechanism, with repeat assessment to evaluate the coagulation response to time or therapeutic intervention. Most studies also reported changes in platelet count and fibrinogen concentration. Only 3 studies assessed components of the hypothesized mechanisms of ATC development by evaluating changes in platelet function (177), response to activated protein C (aPC) blockade (40) and endothelial glycocalyx thickness and plasma syndecan-1 (178).

Methods used to induce coagulopathy.

Iatrogenic hemodilution, hypothermia and/or acidosis were the most common mechanisms utilized to induce deranged coagulation function. Hemodilution prolonged routine coagulation assays and exacerbated hemorrhage from standardized visceral injury (179-185). Induced hypothermia to 33^{o}C in combination with hemodilution was correlated with a further increase in organ bleeding time (186, 187). Isolated hypothermia was only associated with prolonged coagulation assays when the tests were performed at the core body temperature of the animal, with no abnormalities present when performed at the regulation 37^{o}C (188-190). Isolated acidosis was induced via intravenous administration of hydrochloric acid or cross clamping of the aorta following fixed volume hemorrhage to produce ischemia-reperfusion (191-194). It was associated with coagulation dysfunction that failed to improve following reversal with bicarbonate and the effects were compounded by concurrent hypothermia (193-195).

Isolated hemorrhagic shock failed to significantly prolong routine coagulation assays in all but one study in which a 10 fold increase in APTT was observed after 60 minutes of shock (29, 196-198). Isolated tissue trauma was not associated with prolongation of coagulation assays in any study (29, 40). One study utilized an intravenous infusion of tissue factor to simulate the effects of tissue factor release from the site of trauma (199). This produced hyperfibrinolysis, increased D dimer levels and significant prolongation of coagulation assays, although the absence of physical injury limits the clinical applicability of these findings.

The combined effect of trauma and hemorrhagic shock was evaluated in 3 rodent and 7 porcine models with varying effects on coagulation function observed (29, 40, 51, 65, 67, 177, 200-203). All rodent models demonstrated a minimum 20% prolongation in PT, aPTT or PT ratio (PTr) (29, 40, 65) which was attenuated by monoclonal antibodies to aPC in

one study (40). One porcine model demonstrated a brief hypercoaguable response that was followed by significantly reduced viscoelastic function and a 20% prolongation of INR, although a 25% mortality rate was evident within the experimental group (67). A second porcine model demonstrated altered platelet function and prolonged PT that was exacerbated by fluid resuscitation but unaltered by induced hypothermia, although the initial prolongation in PT failed to attain the current clinical definition of ATC (177). Non-significant changes in PT or INR were evident in the remaining 5 porcine models (51, 200-203), although a significant reduction in ROTEM mean clot firmness was evident in one study (203) and significant changes developed following fluid resuscitation and/or the induction of hypothermia and acidosis in the remaining three studies (51, 201, 202).

Discussion

This review identifies a large diversity of animal models utilized to investigate coagulation changes following trauma. This may reflect both the clinical importance of such an area as well as the fact that the pathophysiology of ATC is poorly understood and may be difficult to model accurately. Despite our current understanding of the natural history of traumatic coagulopathy only 10 models attempted to reflect it as an endogenous dysfunction induced by initiating injury and hemorrhage which may be exacerbated by hypothermia, acidosis and hemodilution. Consequently there remains an overall lack of reproducible, clinically relevant models for investigating pathophysiology and evaluating therapeutic interventions.

There are many animal models which have provided information on the effects of hypothermia, acidosis and hemodilution on systemic coagulation function (179-195). Restoration of plasma volume with crystalloid and/or synthetic colloid solutions produces a diffuse microvascular bleeding tendency due to dilution of clotting factors, accelerated endothelial glycocalyx degradation and direct interactions with fibrin polymerization (178, 183, 204). The induction of acidosis to a pH of less than 7.2 via intravenous administration of hydrochloric acid or ischemia-reperfusion injury has been shown to reduce the enzymatic activity of coagulation proteases, producing a coagulopathy that cannot be readily reversed with simple correction of the acidosis (192-194). Induced hypothermia to less than 33^{o}C has similar effects on coagulation protease and platelet function, contributing to coagulation dysfunction and exacerbating existing coagulopathy induced by hemodilution and/or acidosis (188-190, 195). However in the majority of models investigating these insults the hemodilution, hypothermia and/or acidosis precede tissue

injury and hemorrhage. Given our current understanding of the natural history of traumatic coagulopathy the clinical value and relevance of models without initiating tissue trauma and shock is questionable.

Hemodilutional coagulopathy in isolation or in combination with hypothermia was the most common model used to evaluate the therapeutic efficacy of specific hemostatic interventions in trauma. However in the vast majority of these models the resuscitation fluids used to produce hemodilution were not reflective of contemporary resuscitation practices (82, 205). The absence of initiating tissue injury and divergence from current clinical practice may limit translatability of therapeutic information obtained from these models. The effects of spontaneous hypothermia and acidosis are also essentially uncharacterized by current animal models. Changes associated with central cooling induced hypothermia may differ from the spontaneous hypothermia observed in human trauma patients given that they rarely present with a body temperature as low as 33^{o}C (206). The applicability of acidosis induced by hydrochloric acid infusion or ischemia-reperfusion in the absence of tissue trauma and shock is also dubious. The deleterious outcomes associated with acidosis developing in response to tissue hypoxia and trauma may simply reflect the severity of the insult responsible for the acidosis rather than being a direct effect of the acidosis itself.

The mechanism underlying the development of ATC is still incompletely understood. Clinical studies suggest a combination of tissue injury and hypoperfusion as a prerequisite (29, 37). Animal models investigating the effects of isolated trauma and hemorrhagic shock support this, with altered coagulation function in response to isolated insults only evident in one study (196). In contrast animal models of combined trauma and hemorrhage have shown varying degrees of coagulation dysfunction, which may reflect both the severity of the insult used and the intrinsic coagulation function of the animal. The majority of these models have provided information on the presence and time course of coagulopathy in response to trauma and hemorrhage (29, 65, 67, 177, 200, 201). However only 3 models have evaluated aspects of the proposed pathophysiologic mechanisms, and as a result animal models are yet to significantly contribute to the improved mechanistic understanding of ATC (40, 177, 178).

Animal models of trauma enable particular clinical scenarios to be evaluated under controlled conditions and provide opportunities for invasive monitoring and diagnostic

methods that cannot be performed in human trauma patients. Incorporating techniques such as electron microscopy to assess the endothelial glycocalyx, organ microdialysis and sidestream dark field (SDF) camera application to evaluate the microcirculation and immunohistochemistry (IHC) to investigate tissue expression of coagulation proteases in conjunction with a structured coagulation component analysis will facilitate a more sophisticated contribution to the mechanistic understanding of traumatic coagulopathy in future animal models of trauma (137, 207-209). However the use of animals for trauma research also has limitations. The ethical necessities of sedation and anesthesia alter the compensatory physiologic responses to injury and have an unknown effect on the overall response observed (210, 211). Animals are also genetically distinct from humans and demonstrate differences in coagulation assay parameters, coagulation factor concentrations and platelet function that may alter susceptibility to ATC (58-60).

Rodent models are inexpensive and transgenic techniques such as protein C gene mutations provide opportunities to manipulate and evaluate isolated pathways within the coagulation system (212). Rodents have also consistently demonstrated the ability to achieve the clinical definition of ATC in response to trauma and hemorrhage (29, 40, 65). However rodent models are limited by animal size which restricts sample volumes and subsequent assays, hampering the ability to further investigate mechanisms associated with coagulopathy. The genomic response of mice to trauma has also been shown to correlate poorly with humans, bringing the clinical relevance of murine trauma models into question (213).

Pigs are the predominant large animal species that have been used for the investigation of traumatic coagulopathy, which appears appropriate given the established similarities in cardiovascular physiology between pigs and humans (66). However pigs have demonstrated significant variability in coagulation function response to trauma and hemorrhage (51, 67, 177, 200-203). Both viscoelastic and plasma based assays of coagulation function have shown pigs to be relatively hypercoaguable compared to humans and to have protein C levels that are only 36% of human values (58, 60). Given that activation of protein C is hypothesized to be a central driver of ATC, this fundamental difference may impede the ability to model ATC successfully in this species. The only porcine model to achieve the clinical definition of ATC in response to trauma and hemorrhage was associated with a 25% mortality rate (67). This may reflect the severity of the injury required to produce ATC in this species and brings into question the

reproducibility and ethical acceptability of this model. The abundance of porcine models may be limiting information about traumatic coagulopathy that could be gained from other large animal species. Evaluating pathophysiological mechanisms and therapeutic efficacy in a variety of animal species subjected to varying insults and intercurrent treatments would improve the translatability of results and better inform subsequent human trials (214) .

Traumatic insults resulting in tissue damage and uncontrolled hemorrhage occur simultaneously in the human trauma patient. To control the degree of tissue trauma and shock the majority of animal models apply standardized insults in a staggered fashion and utilize either fixed pressure or fixed volume bleeds via an indwelling vascular catheter. The influence of this non-pathophysiologic sequence of insults on overall response is unknown. Fixed pressure hemorrhage controls the degree of hypotension via repeated blood withdrawal and volume replenishment; however it does not mimic the clinical situation and introduces the potential confounders of iatrogenic hemodilution and anti-coagulation (29, 67). Fixed volume hemorrhage allows assessment of the hemodynamic responses to hypotension, although the degree of hypotension is not well defined and variability in the physiologic response is evident (215). Uncontrolled hemorrhage most closely resembles the clinical situation; however it introduces significant physiological variability and risk of animal mortality (189, 216-218). This influences the repeatability of the model and compromises the ethical acceptability by increasing the number of animals required for statistically significant results to be achieved (219, 220).

Existing animal models of trauma and hemorrhage demonstrate considerable variation in the degree of injury severity and target pressure or volume of hemorrhage (29, 40, 51, 65, 67, 177, 200-203). This contributes to inter-model variations in coagulation function response and limits the reproducibility and comparability of these existing models. To improve future models of traumatic coagulopathy the degree of trauma and hemorrhage needs to be titrated to variables that have been shown to correlate with the onset of ATC. Evidence suggests that an injury severity score (ISS) of 25 combined with a base deficit of 6mmol/L is necessary for ATC to develop (29). While the translatability of ISS across species is uncharacterized, future models should aim to produce a reproducible and survivable tissue injury that correlates with an ISS of 25. The degree of hemorrhage should be titrated to a base deficit of 6mmol/L to ensure the necessary degree of tissue hypoxia is achieved consistently, removing the variability that may accompany a fixed

pressure or volume bleed due to differences in pre-experiment fluid balance and physiological response (215). This should improve the clinical relevance and reproducibility of future animal models, facilitating more efficient inter-species comparison and improving the translatability of pre-clinical research.

The presence of ATC was originally characterized as a 50% prolongation of PT, aPTT or INR (22, 23). Subsequent epidemiological work described an INR >1.2 as the clinically significant threshold associated with an increased risk of adverse outcomes (29). However many of these patients show no clinical evidence of prolonged hemorrhage, with abnormalities in coagulation function only evident in laboratory assays of coagulation function (29). It is possible that in these patients abnormal coagulation function develops as a marker of injury severity, which may explain why ATC as defined can be associated with an increased risk of adverse outcomes in the absence of overt clinical coagulopathic hemorrhage.

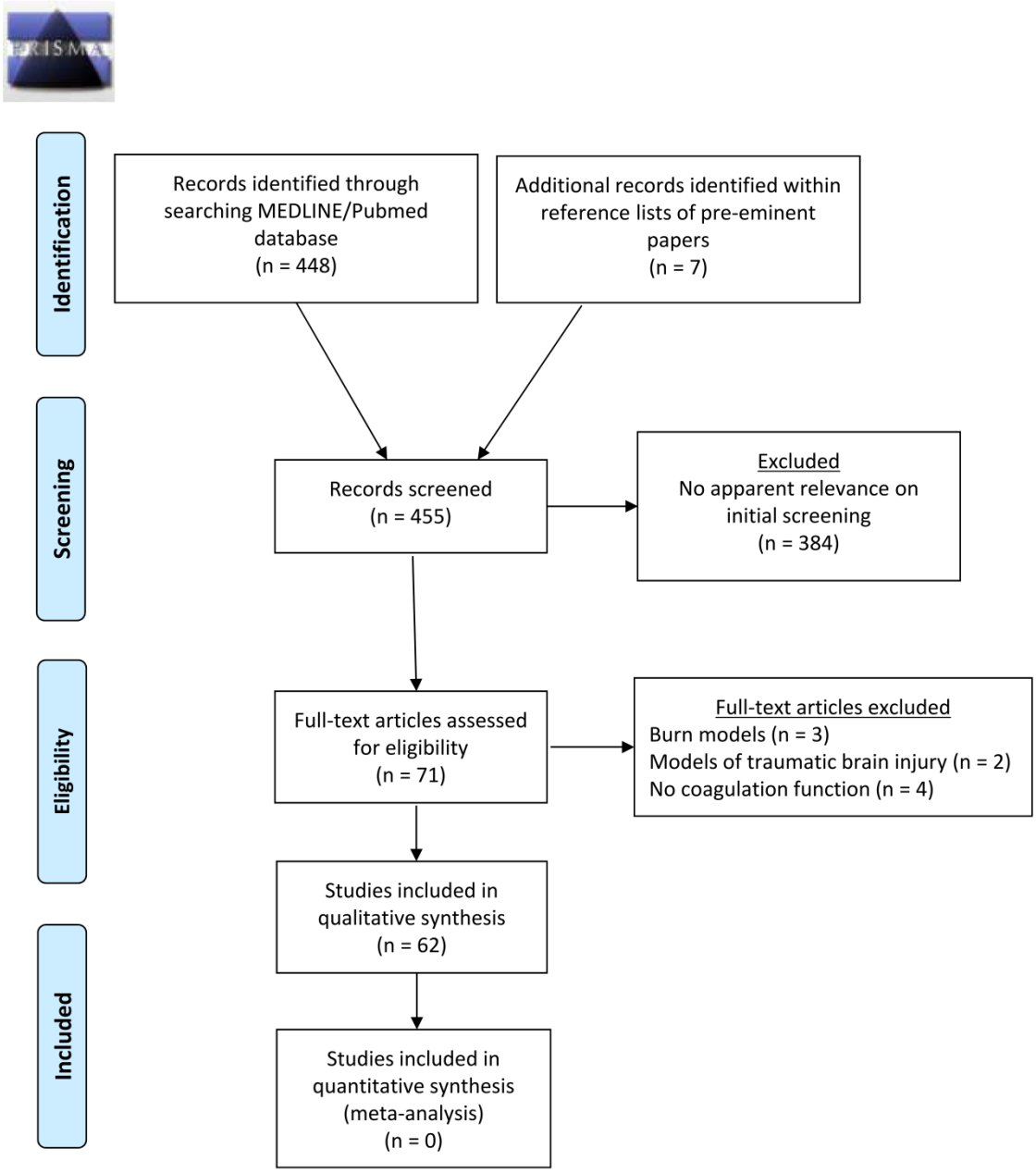
The use of viscoelastic assays to assess coagulation function in animal models of trauma is increasing, which parallels the emerging role of viscoelastic assays in the acute trauma setting. The use of traditional plasma based assays of coagulation function in animal models of trauma is appropriate given that ATC continues to be defined clinically in terms of these assays. However the value of continuing to define ATC in terms of traditional assays has been questioned due to their inability to characterize global coagulation function and delayed availability of results (98). Viscoelastic assays provide a more complete assessment of coagulation function via the evaluation of clot formation, clot strength and fibrinolytic activity and have been validated in a number of animal species (221, 222). However they neglect the contribution of the endothelium, which may continue to be a limitation given the postulated role of the endothelium in the development of ATC. Attempts to define ATC using viscoelastic parameters have been made, as trauma patients with an INR >1.2 show changes in clot formation time, clot strength and fibrinolytic activity (30, 32). Various thresholds have been proposed, however there is no consensus on the viscoelastic definition that should be used (30, 32, 33, 95). Further work is required to ascertain the viscoelastic triggers that identify ATC, and relevant animal models will play an important role in this process.

Conclusion

Traditional animal models of traumatic coagulopathy were inspired by a perceived iatrogenic process and investigated hemodilution, hypothermia and acidosis as the pathophysiological initiators of coagulation dysfunction. The recognition of ATC as an endogenous response to tissue injury and hypoperfusion has altered the perceived etiology and chronology of traumatic coagulopathy. In order to be clinically relevant new animal models should reflect the current natural history of traumatic coagulopathy and utilize a variety of animal species to account for inter-species variation in coagulation function.

Creating a clinically relevant model of trauma and hemorrhage that allows translation of results to humans is difficult given the complexity of the condition and known limitations of animal research. It is likely that no single animal model will answer all questions and utilizing the appropriate model for the question at hand will continue to be a challenge. However preclinical animal studies are necessary to continue improving the management of trauma patients given the limitations of human research in the emergency setting. The development of a valid, reproducible and clinically relevant animal model of trauma is needed to contribute to improved mechanistic understanding of the pathophysiology of ATC and allow future evaluation of novel therapeutic agents in whole biological systems.

Figure 1. PRISMA flow diagram for experimental animal models of traumatic coagulopathy



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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Table 1. Distinct animal models of traumatic coagulopathy published in the last 10 years.

Author	Year of Publication	Animal species	Number of animals per study arm	Method used to induce coagulopathy	Principal coagulation findings	Motivation behind study
Martini WZ et al (195)	2005	Pig	6	Combined acidosis and hypothermia	Increased splenic bleeding time Reduced platelet count Increased PT and aPTT Increased R value on TEG.	Pathophysiology of combined hypothermia and acidosis.
Klemcke HG et al (187)	2005	Pig	18	Combined hypothermia and hemodilution	Increased PT and aPTT Decreased TEG α angle and maximum amplitude Decreased fibrinogen.	Assess effect of recombinant FVIIa
Fries D et al (180)	2005	Pig	14	Hemodilution followed by liver injury	Decreased fibrinogen and platelet count Increased PT and aPTT	Assess effects of fibrinogen concentrate
Martini WZ et al (179)	2006	Pig	6	Hemodilution	Decreased fibrinogen concentration Reduced clotting time and clot strength on TEG.	Pathophysiology of hemodilution
Kiraly LN et al (216)	2006	Pig	15	Grade V liver injury and fluid resuscitation	Increased PT and aPTT Increased alpha angle and clotting index on TEG	Efficacy of Hartman's vs 0.9% saline resuscitation
Martini WZ et al (193)	2006	Pig	7	Isolated acidosis induced by hydrochloric acid infusion	Reduced fibrinogen and platelet count Increased PT, aPTT and ACT Decreased TEG alpha angle and maximum amplitude	Pathophysiology of isolated acidosis
Martini WZ et al (188)	2007	Pig	6	Isolated hypothermia (<33^{o}C)	Increased TEG clotting time and α angle Decreased fibrinogen concentration	Pathophysiology of isolated hypothermia
Kheirabadi B et al (186)	2007	Rabbit	8	Combined hypothermia and hemodilution	Increased PT and aPTT Decreased fibrinogen concentration Decreased TEG alpha angle and amplitude	Evaluate ability of blood tests to detect coagulopathy
Chesebro BB et al (40)	2009	Mice	10	Combined laparotomy and fixed pressure hemorrhage	Increase in aPTT, attenuated by monoclonal antibodies to activated protein C	Contribution of protein C to coagulopathy.
Cho SD et al (201)	2009	Pig	37	Combined femoral fracture and fixed volume haemorrhage, hemodilution, induced hypothermia and induced acidosis	Increased liver bleeding time Prolonged PT/aPTT following hemodilution Reduction in maximum amplitude of clot strength on TEG	Reproducibility of a multi combat model between institutions
Pragst I et al (181)	2010	Rabbit	17	Hemodilution	Increased kidney bleeding time Increased PT time	Efficacy of prothrombin complex concentrate
Frith D et al (29)	2010	Rat	10	Combined laparotomy, femoral fractures and fixed pressure haemorrhage	Increased PTr and aPTTr	Evaluate pathophysiology and definition of ATC

Iwamoto S et al (190)	2010	Rat	12	Hypothermia and hemodilution	Increased ACT	Pathophysiology
White NJ et al (200)	2010	Pig	18	Combined unilateral femoral fracture and fixed pressure hemorrhage	Decreased fibrinogen No significant change in PT or aPTT	Pathophysiology of combined trauma and hemorrhage
Letson HL et al (196)	2012	Rat	10	Isolated fixed pressure hemorrhage	Increased aPTT	Efficacy of 7.5% Na Adenocaine and Mg^{2+}
Mulier KE et al (202)	2012	Pig	8	Combined lung contusion, liver fractures and fixed pressure haemorrhage	Reduced platelet count Increased rate of clot formation and reduced clot stiffness on TEG	Pathophysiology of multi-trauma and haemorrhage
Doran CM et al (51)	2012	Pig	6	Combined blast injury, fixed volume haemorrhage and hemodilution	Increased PT	Evaluate the effect of targeted resuscitation
Lesperance RN et al (192)	2012	Pig	28	Fixed volume haemorrhage and ischemia-reperfusion injury	Increased INR Increased CT, CFT, and reduced α angle and MCF on ROTEM	Assess effects of lactic acidosis on coagulopathy.
Fung YL et al (197)	2013	Sheep	5	Isolated fixed volume haemorrhage	No significant changes in PT or aPTT or any ROTEM parameters	Evaluate the effects of stored blood transfusion
Park KH et al (189)	2013	Rat	8	Hypothermia and uncontrolled hemorrhage from splenic laceration	Increased CT, CFT and reduced α angle and MCF on ROTEM	Pathophysiology
Darlington DN et al (65)	2013	Rat	7	Combined femoral fracture, soft tissue trauma and fixed pressure hemorrhage.	Increased PT and aPTT Decreased MCF on ROTEM	Pathophysiology.
Nishi K et al (218)	2013	Rat	6	Uncontrolled hemorrhage following tail amputation	Prolonged ACT and clot rate following fluid resuscitation	Effects of fluid resuscitation.
Mohr J et al (177)	2013	Pig	10	Blunt chest trauma, liver laceration, fixed pressure hemorrhage and hypothermia	Prolonged PT and decreased Fibrinogen Increased CT, CFT and reduced MCF on ROTEM	Evaluate coagulation function in a multi-trauma model.
Hayakawa M et al (199)	2013	Rat	6	Administration of tissue factor	Reduced platelet count and fibrinogen. Increased PT and D-dimer Reduced anti-thrombin levels.	Effects of tissue factor on coagulation and fibrinolysis.
Hagemo JS et al (203)	2013	Pig	8, 4, 2	Bilateral femoral fractures and soft tissue injury, fixed volume hemorrhage	Decreased fibrinogen and INR. Increased CT on ROTEM	Characterize fibrinogen changes with trauma and hemorrhage.
Martini J et al (183)	2013	Hamster	5	Hemodilution	Decreased MCF and α angle, increased CFT on ROTEM	Evaluate effects of fibrinogen on clotting.

Duan K et al (67)	2014	Pig	16	Right femoral fracture, small intestinal crush and grade III liver injury, fixed pressure haemorrhage	Increased PT and INR Increased R value on TEG.	Characterize changes in clotting function following trauma.

PT=prothrombin time, aPTT=activated partial thromboplastin time, INR=International normalized ratio, TEG=Thromboelastography, ROTEM=thromboelastometry, PTr=prothrombin ratio, aPTTr=activated partial thromboplastin ratio, CT=clotting time, MCF=mean clot firmness, CFT=clot formation time, ACT=activated clotting time

3.3 Discussion of this review article

This systematic review identified 27 distinct animal models developed after 2003 to investigate coagulation dysfunction in trauma. Of these 27 models, 17 still utilised haemodilution, acidosis or hypothermia either alone or in combination as the initiating mechanism for coagulopathy, prior to the creation of tissue injury. This has provided information about the haemostatic effects of these separate insults in complex biological systems. However given our current understanding of the natural history of ATC the ongoing value of models devoid of initiating tissue injury and haemorrhagic shock is limited.

Ten distinct animal models that investigated the combined effect of tissue injury and haemorrhagic shock on coagulation function were identified by this review. Three of these were rodent models, all of which achieved current clinical definitions of ATC using traditional assays of coagulation function (29, 40, 65). Elements of pathophysiology were examined in one of these rodent models, with coagulopathy prevented by the administration of aPC antibodies (40). However further evaluation of alternative pathophysiological mechanisms in these models was restricted by animal size, which limited sample collection and the number of assays that could be performed. The remaining 7 models were porcine models, with considerable inter-model variability in the coagulation function response evident (51, 67, 103, 177, 200-202). No significant change in coagulation function was observed in 6 of these models (51, 103, 177, 200-202). A statistically insignificant reduction in mean clot firmness (MCF), increase in PT and alteration in platelet function was observed in 2 models; however clinical definitions of ATC were not met until after fluid resuscitation had commenced (103, 177). The remaining model is the only porcine model to have achieved clinical definitions of ATC in response to trauma and haemorrhage alone, demonstrating a 20% increase in INR/PT (67). However this model was associated with a 25% mortality rate in the trauma group, creating doubt about the repeatability and ethical acceptability of this model.

This review also highlighted a lack of consensus as to the animal species that may best simulate the human condition. Animals are genetically distinct from humans and demonstrate fundamental differences in coagulation assay parameters, coagulation factor levels and platelet function (58-62). These differences may alter the susceptibility to ATC and influence the translatability of the chosen experimental model. Refinement of future

animal models of ATC requires careful consideration of the chosen animal species. In order to be clinically relevant the chosen animal species must demonstrate more similarities than differences with human cardiovascular physiology and coagulation function.

The reporting of coagulation function testing was mandated as an inclusion criterion for this systematic review, as the overarching aim was to inform the development of an alternative model of traumatic coagulopathy. However the concept of traumatic shock is not limited to coagulation dysfunction, as only a proportion of trauma patients demonstrate impaired coagulation function in response to trauma (22, 29). More broadly traumatic shock encompasses the local and systemic alterations that occur in response to tissue injury, haemorrhage, organ ischaemia, tissue reperfusion and resuscitation (223). It is associated with a post traumatic immune response characterised by an increase in inflammatory mediators and influx of inflammatory cells that predisposes to coagulopathy, sepsis and post-injury multi-organ failure (215, 223). The exclusion of animal models of trauma that failed to describe coagulation function testing is a limitation of this review, as it restricted the ability to fully evaluate additional information regarding traumatic shock that could be obtained from these other models.

3.3.1 The need for an alternative large animal model

Large animal models of coagulopathy are preferable as they facilitate sample collection and allow the application of standard human monitoring techniques (224). Rodent models are more affordable; however the ability to fully investigate pathophysiological mechanisms of coagulopathy *in vivo* is hampered by animal size. Rodents also demonstrate significant differences in coagulation function and the genomic response to trauma when compared to humans, further limiting the clinical relevance of rodent trauma models (58, 213).

As noted in this review, pigs are the only large animal species to have been used to investigate the coagulation function response to trauma and haemorrhage. This may appear appropriate given the number of physiological similarities pigs share with humans (66). However pigs demonstrate significant differences in coagulation function, which may impact the ability to successfully model ATC in this species. Pigs are hypercoaguable compared to humans on both traditional plasma based assays and viscoelastic assays of coagulation function, and have significantly lower levels of protein C (58, 60). This may

explain why pig models fail to demonstrate the degree of ATC seen in rodents despite the use of similar protocols. The use of an alternative large animal species may therefore improve understanding of coagulopathy in trauma and better inform subsequent human studies.

Sheep may represent the ideal candidate animal, as they share many similarities in haemodynamic, microcirculatory and immunologic function with humans (75-77). Sheep have been widely used in biomedical research to model other human pathologies including asthma (68, 69), myocardial reperfusion (73), burn injury (225), haemophilia (226), osteoporosis (70), sepsis (71, 74, 227) and various presentations of acute lung injury (ALI) (72, 77, 228). Comparative studies of human, porcine, rodent, canine and ovine coagulation function using routine coagulation tests, ROTEM and clotting factor assays also indicate that the human coagulation system demonstrates the greatest similarity with that of sheep (58, 59).

3.3.2 Animal model design

Considerable variability in the type of injury and method of haemorrhage was evident in the models of trauma and haemorrhage identified by this review. Hind-limb fractures formed the basis of tissue injury in all models, combined with varying combinations of soft tissue contusions (103, 200) blast injuries (51), intestinal crush injuries (65, 67), laparotomy incisions (29, 40) and liver injuries (67, 177, 202) to increase the degree of tissue trauma. Fixed pressure haemorrhage was utilised in the majority of models (29, 40, 65, 67, 177, 200, 202), although there was no apparent agreement with regards to the desired target pressure. This lack of consensus in the type of injury and degree of haemorrhage has contributed to inter-model variations in haemodynamic and coagulation function response, limiting the reproducibility and clinical relevance of these existing models. Targeting the severity of injury and type of haemorrhage to variables that correlate with the development of ATC may facilitate the development of a more clinically relevant, reproducible and ethically acceptable model.

3.3.2.1 Severity of injury

Retrospective human studies indicate that an injury severity score (ISS) of more than 15 is a necessary condition for the development ATC, with the majority of cases associated with an ISS > 25.(29, 39, 92, 229). The ISS was first introduced in 1974 and continues to be one of the most widely used measures of injury severity (230). It is based upon an

abbreviated injury score (AIS) which is an anatomic scoring system used to describe the site of injury, force applied and extent of damage (231). The ISS was introduced to take into account the contribution of second and subsequent injuries to morbidity and mortality and is calculated by assigning injuries to one of six body regions (230). The severity of each injury is then ranked on a scale of 1 (minor) to 5 (critical) (231). The three highest scores are then squared and added together to produce the ISS (table 3).

The translatability of the ISS across species is yet to be characterised. However creating a survivable and reproducible tissue injury that correlates with an ISS of 25 may improve the clinical relevance of future models. Hind limb fractures would appear to be an appropriate basis for the tissue injury, however are insufficient in isolation as they only result in an ISS of 9 (table 3). Additional injuries are required to meet the proposed ISS, and the chosen injury needs to minimise potential confounders of uncontrolled haemorrhage, endotoxaemia and tissue ischaemia. Pulmonary contusions have been utilised in an ovine model investigating the effects of fat emboli and acute respiratory distress syndrome (ARDS) following femoral fracture, and have been associated with a low mortality rate (232). As pulmonary contusions make a significant contribution to ISS, the use of pulmonary contusions in conjunction with hind limb factures may facilitate the creation of a reproducible and survivable tissue injury that has an ISS of 25 (table 3).

Table 3. *Example calculation of an ISS*

Region	**Injury Description**	**AIS**	**Highest AIS[2]**
Head and Neck	No injury	0	
Face	No injury	0	
Chest	Multiple lung contusions Chest wall laceration	4 2	16
Abdomen	No injury	0	
Extremity	Displaced compound tibial fracture (upper third)	3	9
External	No injury	0	0
Injury Severity Score			25

3.3.2.2 Type of haemorrhage

Retrospective human studies indicate that in the presence of severe tissue injury a base deficit of 6mmol/L is associated with the onset of ATC (29). This is thought to reflect systemic hypoperfusion developing secondary to haemorrhagic shock. In the human trauma patient haemorrhagic shock develops in response to uncontrolled haemorrhage, thus the use of uncontrolled haemorrhage in experimental models would most closely replicate the clinical situation. However uncontrolled haemorrhage introduces significant physiological variability, compromising the reproducibility of the model (189, 218). It also increases the risk of intra-experimental animal mortality, which would in turn impact on the ethical acceptability of the model.

Two options for the creation of controlled haemorrhagic shock were identified by this review: fixed volume or fixed pressure haemorrhage. Fixed pressure haemorrhage is more commonly utilised by existing trauma and haemorrhage models, as it allows the degree and duration of hypotension to be controlled (224). However in order to maintain the mean arterial pressure at the target level, repeated blood withdrawal and volume replacement with crystalloid solutions or anticoagulated blood is required (29, 65, 67, 200). This increases the likelihood of iatrogenic haemodilution or anti-coagulation, and is not reflective of the clinical situation in the human trauma patient. Fixed volume haemorrhage offers an alternative, as it allows the degree of haemorrhagic shock to be controlled whilst facilitating assessment of the haemodynamic responses to hypovolaemia (224). This is more reflective of the clinical situation seen with uncontrolled haemorrhage; however it does increase the potential for physiological variability.

3.3.3 Summary

An alternative large animal model of coagulopathy is desirable. Pigs are the only large animal species that have been used to evaluate the haemostatic response to trauma and haemorrhage. However recognised differences in coagulation function compared to humans appear to be limiting the ability to successfully model ATC in this species. Sheep demonstrate similarities in haemostatic, haemodynamic and microcirculatory function with humans, suggesting they may be the ideal candidate animal in which to develop an alternative large animal model of ATC. Combining fixed pressure haemorrhage with a reproducible tissue injury that equates to an ISS of 25 may facilitate the development of a more clinically relevant animal model of ATC, which may improve understanding of the condition and facilitate future evaluation of alternative therapeutic strategies.

CHAPTER 4: ACTIVATION OF THE PROTEIN C PATHWAY AND ENDOTHELIAL GLYCOCALYX SHEDDING IS ASSOCIATED WITH COAGULOPATHY IN AN OVINE MODEL OF TRAUMA AND HAEMORRHAGE.

van Zyl N, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SR, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and hemorrhage*. J Trauma Acute Care Surg 2016; 81(4):674-684

4.1 An introduction to this peer reviewed publication

This manuscript addresses the second and third aims of this thesis: to develop an ovine model of trauma and haemorrhage that demonstrates coagulation changes consistent with current definitions of ATC, and to observe the relationship between coagulopathy and the protein C pathway, endothelial glycocalyx, platelet function and fibrinolysis within this model.

The information obtained from the systematic review discussed in Chapter 3 facilitated the design of the large animal model utilised in this manuscript. A need for an alternative large animal model was identified, with sheep chosen for their similarities with humans with regards to size, cardiovascular physiology and coagulation function, and the experience of the research group with ovine models of critical illness. Controlled haemorrhagic shock was created using fixed volume haemorrhage to enable assessment of the physiologic responses to hypovolaemia. Bilateral tibial fractures were combined with pulmonary contusions to produce a repeatable tissue injury that equated to an ISS of 25. A graded degree of injury severity was also assessed in this thesis through the inclusion of two different injury groups. The moderate injury group underwent bilateral tibial fractures, single left upper and left lower lung lobe contusions and haemorrhage of 20% blood volume. The severe injury group underwent bilateral tibial fractures, bilateral hamstring crush injuries, two left upper and left lower lung lobe contusions and haemorrhage of 30% blood volume. Grading the degree of trauma in this way allowed the effects on base deficit

and coagulation function to be assessed in a step wise manner in the hope that a more reproducible, clinically relevant and ethically acceptable animal model would result.

The development of coagulopathy in this model was assessed using both traditional and viscoelastic assays of coagulation function, and the coagulation changes displayed by the animals are outlined in this manuscript. It was considered necessary to demonstrate coagulopathy consistent with INR (29), aPTT (23) and ROTEM (30) based definitions in order to validate this model as clinically relevant . This was achieved, with the severe trauma group of animals developing changes in INR, aPTT and EXTEM A10 that were comparable with current clinical definitions of ATC.

The proposed pathophysiological contributors to ATC were then evaluated in this model. Changes in the protein C pathway were assessed by evaluating changes in sTM, protein C, aPC, factor V, factor VIII and PAI-1. A significant association between the protein C pathway and coagulopathy was evident, with coagulopathy correlated with an increase in sTM and aPC and a reduction in protein C, factor VIII and PAI-1. The contribution of the endothelial glycocalyx to ATC was assessed by evaluating changes in syndecan-1 and hyaluranon, which have been utilised as markers of endothelial glycocalyx damage in human studies. Coagulopathy was correlated with an increase in both syndecan-1 and hyaluranon in this model, further supporting the hypothesised contribution of endothelial glycocalyx shedding to ATC. Fibrinolysis was assessed in this model by evaluating changes in D-dimer levels, FIBTEM parameters. Platelet function was evaluated using impedance aggregometry (Multiplate analyser) with collagenase and ADP agonists. There was no evidence of significant fibrinolysis or altered platelet function to suggest a contribution to coagulopathy in this model.

4.2 Reprint of accepted manuscript

Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and hemorrhage.

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Abstract

Introduction: Acute traumatic coagulopathy (ATC) is an endogenous coagulopathy that develops following tissue injury and shock. The pathogenesis of ATC remains poorly understood, with platelet dysfunction, activation of the protein C pathway and endothelial glycocalyx shedding all hypothesized to contribute to onset. The primary aim of this study was to develop an ovine model of traumatic coagulopathy, with a secondary aim of assessing proposed pathophysiological mechanisms within this model.

***Methods*:** Twelve adult Samm-Border Leicester cross ewes were anesthetized, instrumented and divided into three groups. The moderate trauma group (n=4) underwent 20% blood volume hemorrhage, bilateral tibial fractures and pulmonary contusions. The severe trauma group (n=4) underwent the same injuries, an additional hamstring crush injury and 30% blood volume hemorrhage. The remaining animals (n=4) were uninjured controls. Blood samples were collected at baseline and regularly post injury for evaluation of routine hematology, arterial blood gases, coagulation and platelet function, factor V, factor VIII, plasminogen activator inhibitor-1, syndecan-1 and hyaluranon levels.

***Results*:** At 4 hours post injury a mean increase in international normalised ratio (INR) of 20.50±12.16% was evident in the severe trauma group and 22.50±1.00% in the moderate trauma group. An increase in activated partial thromboplastin time (aPTT) was evident in

both groups, with a mean of 34.25±1.71s evident at 2 hours in the severe trauma animals and 34.75±2.50s evident at 4 hours in the moderate trauma animals. This was accompanied by a reduction in ROTEM EXTEM A10 in the severe trauma group to 40.75±8.42mm at 3 hours post injury. Arterial lactate and indices of coagulation function were significantly correlated (R=-0.86, p<0.0001). Coagulopathy was also correlated with activation of the protein C pathway and endothelial glycocalyx shedding. Whilst a significant reduction in platelet count was evident in the severe trauma group at 30 minutes post injury (p=0.018) there was no evidence of altered platelet function on induced aggregation testing. Significant fibrinolysis was not evident.

Conclusions: Animals in the severe trauma group developed coagulation changes consistent with current definitions of ATC. The degree of coagulopathy was correlated with the degree of shock, quantified by arterial lactate. Activation of the protein C pathway and endothelial glycocalyx shedding were correlated with the development of coagulopathy; however altered platelet function was not evident in this model.

Keywords*:* Acute traumatic coagulopathy, ovine, protein C

Background

Severe hemorrhage remains the leading cause of preventable death in trauma, responsible for up to 40% of trauma related mortality (3). Efforts to achieve hemostasis are complicated by a trauma induced coagulopathy (TIC), which was traditionally attributed to the loss, dilution and dysfunction of coagulation proteases secondary to hemodilution, hypothermia and acidosis (20). However it is now recognized that a mechanistically distinct acute traumatic coagulopathy (ATC) develops independent of these factors and is present in 24-41% of trauma patients at hospital admission (22, 23).

Multiple definitions of ATC have been proposed; however there remains no consensus regarding the laboratory definition that should be used. ATC was originally characterized as a 50% prolongation in international normalised ratio (INR) or prothrombin time (PT), or an activated partial thromboplastin time (aPTT) > 34s (22, 23). Subsequent epidemiological work has found a 20% prolongation of INR to be clinically significant for mortality and blood product transfusion (29). More recently attempts have been made to define ATC using both thromboelastometery (ROTEM) or thromboelastography (TEG). A clot amplitude < 35mm at 5 minutes (A5), <40mm at 10 minutes (A10), reaction time (R value) >1.1 seconds and K time >2.5 seconds have all been associated with increased

transfusion requirements and mortality in trauma (30-34). However there remains no universally accepted viscoelastic definition of ATC.

A combination of tissue injury and tissue hypoperfusion appears necessary for ATC development (29, 37, 39). However the exact causative mechanisms remain poorly understood. Protein C activation may play a central role, with clinical studies demonstrating an association between protein C reduction, coagulopathy and mortality (37-39). One theory is that pathological activation of protein C results in systemic anticoagulation and hyperfibrinolysis through the inactivation of factors Va, VIIIa and plasminogen activator inhibitor-1 (PAI-1) (37, 38, 40). However the validity of this hypothesis has been questioned by recent *in vitro* work showing that factor Va pools are resistant to cleavage by activated protein C (aPC) at the concentrations observed in trauma patients (44). It has also been demonstrated that PAI-1 circulates at roughly ten times the concentration of aPC, raising doubts about the ability of aPC to deplete PAI-1 levels to the extent required to accelerate fibrinolysis (41). Consequently it has been suggested that increased tissue plasminogen activator release may drive fibrinolysis, and that ATC should instead be classified as a form of disseminated intravascular coagulation (DIC) with a fibrinolytic phenotype (43, 44). There is also emerging evidence to suggest that platelet dysfunction and shedding of the endothelial glycocalyx may contribute to ATC, although the exact contributions of these factors remain unclear (45-48).

There are several published porcine models that have attempted to characterize changes in coagulation function secondary to trauma and hemorrhage (63). However few have achieved proposed definitions of ATC, and evaluation of pathophysiological mechanisms was limited in most cases (63). Comparative studies of coagulation function have shown pigs to be relatively hypercoaguable compared to humans, which may impede the ability to model ATC in this species (58, 60). The effect of non-traumatic hemorrhage of 30% blood volume on coagulation function has been evaluated in sheep (197); however there are no published ovine models evaluating the combined effects of trauma and hemorrhage . Sheep share a number of similarities in cardiorespiratory and hemostatic function with humans, demonstrating equivalent values for fibrinogen, aPTT, and ROTEM parameters (58, 59, 76). These similarities suggest that they may be a suitable species in which to develop an alternative large animal model of ATC.

This study describes a model development project, with the primary aim of creating an ovine model of trauma and hemorrhage that developed coagulation changes consistent with ATC as defined by aPTT, INR and ROTEM. The secondary aim was to observe associations between coagulopathy, the protein C pathway, fibrinolytic system, endothelial glycocalyx and platelet function within this model.

Materials and Methods

This study was approved by the University Animal Research Ethics Committee of both the Queensland University of Technology and University of Queensland. Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific purposes. Animals were housed in a dedicated facility with ad libidum access to food and water. Twelve female 2 year old Samm Border-Leicester cross sheep (weight 43.9 ± 1.2kg) were used in the study.

Instrumentation and Anesthesia

Prior to surgical intervention all animals underwent a routine health check and were fasted for 12 hours. A triple lumen central line (Arrow, PA, USA) was placed in the right internal jugular vein for drug delivery. Two 8.5 Fr percutaneous sheath introducers (CCombo, Edwards Lifesciences, Singapore) were placed in the left jugular vein for hemorrhage and pulmonary artery catheter placement. Anesthesia was induced with intravenous (IV) buprenorphine (0.01mg/kg), midazolam (0.5mg/kg) and alfaxalone (3mg/kg) and maintained with continuous rate infusions of alfaxalone (6mg/kg/hr), midazolam (0.25mg/kg/hr), fentanyl (15µg/kg/hr) and ketamine (10mg/kg/hr) with rates titrated to maintain surgical anesthesia. Animals were intubated and ventilated with a Galileo ventilator set to 12 breaths/minute, a tidal volume of 10ml/kg and 21% FiO_2 (233). Electrocardiogram and pulse oximetry monitoring was commenced. A 20G cannula was placed in the left facial artery (Leadercath, Vygon, UK) for arterial blood pressure monitoring and sampling. A pulmonary artery catheter was placed via the proximal left jugular sheath for monitoring of continuous cardiac output (CCO), mixed venous oxygen saturation (SvO_2) and temperature via an Edwards Vigilance Monitor (Edwards Lifesciences, CA, USA), with normothermia maintained throughout. All animals received a 500ml bolus of lactated Ringer's solution followed by a 3ml/kg/hr infusion during the instrumentation period to replace estimated third space and salivary losses (234). Splenic ligation was performed to attenuate physiological differences in the red cell storage capacity of the ovine spleen. Splenic contraction in humans has minimal effect on

circulating red cell mass; however splenic contraction in response to physiological stress in sheep can increase circulating red cell mass by up to 26% (235, 236). Ligation was performed via a paracostal approach with intrasplenic administration of 0.2ml of 1:10,000 epinephrine prior to ligature placement to promote splenic contraction (237), maximizing the circulating red cell mass prior to hemorrhage. Bilateral femoral nerve blocks were performed using 20ml of 0.25% bupivacaine for regional anesthesia of the hind limbs.

Experimental interventions.

At the completion of instrumentation sheep were allocated to one of three groups: uninjured controls (n=4), moderate trauma (n=4) or severe trauma (n=4). Sample size was determined using a longitudinal power calculation, giving 80% power to detect a difference in EXTEM A10 of ≥5mm per hour between the groups. This calculation was based upon EXTEM A10 as ovine reference ranges and variability have been established, and it has been shown to be a sensitive predictor of coagulopathy in trauma (30, 59). Crystalloid administration was reduced to 10ml/hr and the animals underwent an injury phase. Standardized 2cm x 2cm left lung lobe contusions were created through a left thoracotomy using a custom made pneumatic device calibrated to 15 psi (232). The moderate trauma sheep received single upper and lower lobe contusions, whilst the severe trauma sheep received two upper and lower lobe contusions. A 20 Fr Argyle chest drain was inserted into the pleural space and the thoracotomy closed. A custom built guillotine device weighted to 8.2kg and released from a height of 1.2 meters was used to create bilateral tibial fractures (238). A standardized soft tissue injury was created in the hamstring region of the severe trauma sheep using a custom made pneumatic vice calibrated to 120psi. Controlled hemorrhage was performed over a 10 minute period via gravity assisted drainage through the distal sheath in the left jugular vein to a target of 20% blood volume (moderate trauma sheep) or 30% blood volume (severe trauma sheep), with blood volume calculated as 65ml/kg (239). Following completion of the injury phase all groups were monitored for 6 hours with no further intervention and then euthanized with 10ml pentobarbitone sodium (325mg/ml).

Sample collection and storage

Blood samples were collected into EDTA, 3.2% sodium citrate, lithium heparin and hirudin blood tubes at baseline, 30 minutes post injury, then hourly post injury until completion of the 6 hour monitoring period. Samples were separated and stored at -80^{o}C pending analysis. ROTEM, platelet function and hematology were performed on whole blood within

30 minutes of collection. Post mortem tissue samples were collected from the lungs, myocardium, left kidney and liver and fixed in 10% formalin. All assays were performed according to manufacturer's instructions.

Coagulation function and hematology analysis

Citrated plasma was analyzed for PT, aPTT, D-dimer levels and fibrinogen (Clauss) on the ACL TOP analyzer (Werfen, Sydney, Australia). Platelet function was assessed on the Multiplate analyzer (Haemoview Diagnostics, Brisbane, Australia) using collagen and adenosine diphosphate (ADP) agonists, as trauma patients demonstrate changes in platelet aggregometry with these agonists, ovine reference ranges for these agonists exist, and ovine platelets have proven unresponsive to TRAP-6, arachidonic acid and risocetin agonists (48, 59). Thromboelastometry (EXTEM, INTEM and FIBTEM assays) was performed on the ROTEM (Haemoview Diagnostics). Full blood evaluation was performed using the veterinary mode of the Act Diff hematology analyzer (Beckman Coulter Australia Pty Ltd).

Blood component analysis

Quantitative measurements of factor V (FV), factor VIII (FVIII), protein C and PAI-1 were performed on citrated plasma using the ACL TOP analyzer; with protein C values quantified using a chromogenic assay. Soluble thrombomodulin (sTM) and aPC levels were measured in duplicate on EDTA plasma using commercially available immunoassays (Sheep soluble thrombomodulin ELISA kit, Mybiosource, San Diego USA; Sheep activated protein C ELISA kit, BlueGene lifesciences, Shanghai, China)

Metabolic analysis

Arterial blood samples were analyzed for pH, lactate (mmol/L), base excess/deficit (mmol/L) and bicarbonate (mmol/L) using an automated blood gas analyzer (ABL System 625, Radiometer, Denmark). Plasma catecholamine levels were measured using a Waters 2695 Separation Module (Waters Corporation, NSW, Australia).

Endothelial glycocalyx evaluation

Endothelial glycocalyx breakdown products were measured in duplicate using commercially available immunoassays in serum (Sheep syndecan-1/CD138 ELISA kit, Mybiosource, San Diego USA; Hyaluronan ELISA, Echelon Biosciences, Salt Lake City, USA)

Histology

Formalin fixed tissue samples underwent routine processing and paraffin embedding. 4µm sections were cut and stained with hematoxylin and eosin. Slides were analyzed for fat emboli and histological evidence of DIC (240).

Statistical analysis

Normally-distributed continuous data measurements were described using the mean and standard deviation. The mean values were grouped by time points and compared for significant differences over time within the experimental groups using a two-way repeated measures analysis of variance (ANOVA). Tukey Kramer significance adjustment was used to compare significant differences between groups. The relationship between data sets was evaluated using a two tailed Pearson correlation. Significant differences were defined as a two tailed p value <0.05. All statistical analyses were performed using GraphPad PRISM 6 for Windows (GraphPad Software, San Diego, USA).

Results

Hemodynamic changes

Changes in hemodynamic parameters are depicted in figure 1. At baseline all variables were comparable between the three groups. Immediately following trauma and hemorrhage MAP decreased significantly from baseline in both the moderate trauma (116.5±4.04 to 41.25±8.60mmHg, $p<0.001$) and severe trauma (113±7.20 to 30.50±4.04mmHg, $p<0.001$) groups, remaining significantly lower than the control group mean throughout the monitoring period. The mean CCO decreased from baseline immediately following injury in both the moderate trauma (4.5±0.58 to 3.5±0.48L/min, $p<0.01$) and severe trauma (4.25±0.96 to 3.25±0.34L/min, $p<0.001$) groups, returning to baseline levels by 4 hours. A significant rise in heart rate was evident from 1.5 hours post injury in both the moderate trauma (82.5±7.6 to 113.5±13.0 bpm, $p=0.035$) and severe trauma (79.0±2.6 to 116.8±19.4 bpm, $p=0.033$) groups.

Hematology and metabolic function

Hematology and metabolic parameters are summarized in table 1. All groups demonstrated an initial rise in mean hemoglobin secondary to induced splenic contraction. Following hemorrhage a significant reduction in mean hemoglobin was evident in both trauma groups compared to the control group mean ($p<0.001$). The platelet count in the

severe trauma group was lower than control from 30 minutes post injury (p=0.018), however there were no significant differences in ADP or collagen-induced platelet aggregation between the groups. Lactate rose significantly at 1 hour post injury in the severe trauma group (p=0.017) and at 4 hours post injury in the moderate trauma group (p=0.011). This was accompanied by a significant reduction in base excess in the severe trauma animals from 1-3 hours post injury, however was not associated with the development of a base deficit or acidosis. The high bicarbonate levels in all animals suggest this may have been due to the development of a co-existing metabolic alkalosis of unknown cause.

Coagulation function.

Changes in coagulation function are summarized in table 2. Significant prolongation of aPTT was evident from 30 minutes post injury in the severe trauma group (p=0.002) and 1 hour post injury in the moderate trauma group (p=0.011). A significant elevation in INR was evident in both trauma groups at 5 hours post injury, with a mean increase from baseline of 20.50±12.16% in the severe trauma group and 22.50±1.00% in the moderate trauma group at 4 hours post injury (figure 2). Fibrinogen decreased significantly in the severe trauma group compared to controls at 4 hours post injury (p=0.037). EXTEM A10 was significantly reduced in the severe trauma group from 3 hours post injury (figure 2). The severe trauma group also demonstrated a significant reduction in INTEM A10 (p=0.01) and INTEM α angle (p=0.015) compared to controls at 5 hours post injury. FIBTEM measurements were not significantly different between groups.

Coagulation component and endothelial glycocalyx analysis

Changes in coagulation proteases and endothelial glycocalyx markers are summarized in table 3. FVIII levels were reduced in the severe trauma group compared to controls from 30 minutes post injury (p=0.002). The severe trauma group also demonstrated a significant reduction in FV levels from baseline over time (p<0.0001), however when compared to the control group a statistically significant difference was not evident. Protein C levels decreased significantly in the severe trauma group compared to control from 1 hour post injury (p=0.06), with a significant elevation in aPC levels evident from 3 hours. sTM levels were increased in the severe trauma group from 1 hour post injury (p<0.0001) and PAI-1 levels were reduced from 30 minutes post injury (p=0.006). No significant change in D-dimer levels was evident. Syndecan-1 levels were significantly elevated in the severe trauma group compared to the control group from 1 hour post injury (p=0.018),

and hyaluronan significantly higher from 3 hours post injury (p=0.018). There was no significant change in plasma catecholamine levels evident.

Histology

There was no evidence of fat emboli and no evidence of perivascular hemorrhage, microthrombi, microinfarction, fibrin exudation or hyaline membrane formation in any tissue to suggest DIC.

Correlations between coagulation function, metabolic function, coagulation protease and endothelial glycocalyx parameters in the severe trauma grop.

Pearson correlation coefficients are outlined in table 4. Coagulopathy as defined by EXTEM A10 and aPTT was strongly correlated with arterial lactate levels ($p<0.0001$). Correlation between EXTEM A10, fibrinogen concentration and platelet count was evident, with the reduction in fibrinogen and platelet count also correlated with the decrease in INTEM alpha angle. Changes in the protein C pathway were correlated with coagulopathy, with the reduction in protein C, FVIII and FV correlated with both aPTT and EXTEM A10. The rise in sTM and reduction in PAI-1 was also correlated with EXTEM A10, with the rise in aPC correlated with aPTT. Both protein C and aPC were correlated with changes in INTEM alpha angle, FVIII, FV, PAI-1 and sTM. EXTEM A10 was also strongly correlated with the increase in syndecan-1 and hyaluranon. Syndecan-1, hyaluranon and sTM levels were correlated with the rise in arterial lactate concentration.

Discussion

This study presents the first ovine model of coagulopathy in response to trauma and hemorrhage, with coagulation changes in the severe trauma group consistent with current definitions of ATC. An increase in aPTT to 34.25 ± 0.85 s was evident at 2 hours post injury, EXTEM A10 had decreased to 40.75 ± 4.21mm at 3 hours post injury and INR had increased by 20.50 ± 6.08% at 4 hours post injury (23, 29, 30). The model was designed to simulate an injury severity score (ISS) of 25 based upon evidence suggesting an ISS of 25 is necessary for ATC to develop, and was combined with fixed volume hemorrhage to allow assessment of the hemodynamic responses to hypovolaemia (29). A graded severity of hemorrhage was used to facilitate development of a survivable insult that achieved the desired end points, with a maximum volume of 30% based upon a previous study of non-traumatic hemorrhage (197). Tissue hypoperfusion was evidenced by the

rise in plasma lactate and was correlated with the onset of coagulopathy, further supporting the importance of tissue hypoperfusion in the development of ATC (29).

Activation of the protein C pathway has been considered instrumental to ATC, with the accompanying protein C depletion postulated to contribute to the increased risk of post injury multi-organ failure and mortality (37, 38). Mechanistic evaluation in a murine model of ATC further supports this theory, with the onset of coagulopathy prevented by the administration of aPC antibodies (40). Protein C is activated by thrombin bound concurrently to thrombomodulin and the endothelial protein C receptor (37). It is hypothesized that hypoperfusion results in increased thrombomodulin release from the endothelium, diverting thrombin away from clot formation towards the activation of protein C (37, 38, 40). aPC then inhibits FVa and FVIIIa, with the anti-coagulant effects of aPC mediated primarily through the inactivation of FVa (44). In this study the onset of coagulopathy was strongly associated with activation of the protein C pathway. The severe trauma animals showed a significant rise in sTM that was correlated with lactate levels, supporting the role of hypoperfusion in increasing thrombomodulin release. ROTEM alpha angle is reflective of the thrombin burst, with the reduction in INTEM alpha angle in the severe trauma animals consistent with reduced thrombin activity. This was correlated with the rise in sTM and aPC, suggesting diversion of thrombin towards protein C activation. Inhibition of the coagulation cascade was suggested by the strong correlations between changes in aPC, FV and FVIII. The development of global anti-coagulation was supported by the strong correlation between changes in protein C, aPC, FV, FVIII, sTM and coagulopathy as defined by both EXTEM A10 and aPTT. This conflicts with published *in vitro* findings suggesting that FVa pools are resistant to aPC cleavage at the levels present in trauma patients (44). However correlation is not proof of causation, and given the primary aim of this study was the development of an ovine model of traumatic coagulopathy no mechanistic experiments were undertaken. Future mechanistic studies in this model once refined and established are indicated to better evaluate the contribution of the protein C pathway.

A hyperfibrinolytic state has been reported to play an important role in ATC (89, 114). The mechanism behind fibrinolysis is unclear, with PAI-1 inhibition, tissue factor release and t-PA production proposed as triggers (89, 199). In this study inhibition of PAI-1 was suggested by a reduction in PAI-1 levels that was correlated with the increase in aPC. The reduction in PAI-1 was also correlated with the reduction in fibrinogen and EXTEM

A10 values, suggesting loss of fibrinogen may have contributed to coagulopathy in this model. However there was no alteration in D-dimer levels or FIBTEM parameters to suggest the development of overt fibrinolysis, which is consistent with published findings suggesting aPC is unable to inhibit PAI-1 to the extent required to produce clinically relevant fibrinolysis (41). As a gradual decline in fibrinogen levels was also evident in the control animals in this study it is possible that endogenous hemodilution, repeated blood sampling and anesthesia may have contributed to the fibrinogen depletion observed. It has also been proposed that ATC is actually a fibrinolytic form of DIC, as the initial changes are positive on International Society on Thrombosis and Hemostasis (ISTH) DIC scoring systems (129). This appears unlikely given the absence of histological features of DIC in patients with ATC (43, 129), which was further supported by the lack of typical histopathological findings in this study.

The endothelial glycocalyx is a negatively charged anti-adhesive and anti-coagulant surface layer that protects the endothelial cells and maintains vascular barrier function (135). Traumatic injury and shock result in tissue ischemia, activation of the inflammatory system and a catecholamine surge which may lead to endothelial cell activation and glycocalyx degradation (45, 132, 134). Emerging evidence suggests that these factors may play a role in ATC, with endothelial glycocalyx shedding associated with coagulopathy and mortality in trauma patients (45, 46, 132). Degradation of the glycocalyx has been shown to increase local thrombin generation and fibrinolysis, which in the presence of increased sTM from damaged endothelial cells may enhance protein C activation (135). A volume of plasma containing heparin like substances is also held within the glycocalyx, and release of this following degradation may lead to direct anti-coagulant effects from endogenous heparinisation (46). Syndecan-1 and hyaluranon have both been utilized as markers of glycocalyx degradation in human studies (45, 133). In this study both markers were correlated with EXTEM A10, supporting the hypothesized contribution of glycocalyx degradation to coagulopathy. Rodent models of ATC suggest catecholamine excess may underlie glycocalyx degradation, with coagulopathy and glycocalyx shedding prevented by sympathetic blockade (241, 242).However this model failed to demonstrate an association between glycocalyx degradation and catecholamine excess. Given the correlation between lactate, syndecan-1 and hyaluranon in this study it would appear that ischemia secondary to hypoperfusion is the more likely trigger for glycocalyx degradation in trauma.

Dysfunction of platelet activation and adhesion pathways is apparent in severe trauma patients; however the exact contribution to ATC remains unclear (47, 243). The severe trauma animals in this study developed thrombocytopenia post injury, consistent with human findings in ATC (47), which was correlated with reductions in EXTEM A10 and INTEM alpha angle. This suggests thrombocytopenia may have contributed to reduced clot formation and clot strength, although there was no change in induced platelet aggregation parameters to indicate altered function in this model. However it has been demonstrated that the magnitude of ADP and collagen-induced platelet aggregation in sheep is less than half of that in humans, which may have contributed to the lack of significant findings in this model (59).

Any conclusions drawn from this study should be tempered by several inherent limitations. In human casualties tissue damage and hemorrhage occur simultaneously. To facilitate control over the degree of tissue injury and shock this model applied standardized insults in a staggered fashion, and the influence of this non pathophysiologic sequence on overall response is unknown. The ethical necessities of sedation and anesthesia alter the compensatory physiologic responses to injury by reducing sympathetic activation, and have an unknown impact on the overall response (211). Similarities between the ovine and human hemostatic systems have been demonstrated (58, 59); however there are acknowledged differences in primary and secondary hemostasis that may impact the translatability of findings (59). In human patients ATC is evident within 30 minutes of injury (22, 37, 89). In this study the severe trauma animals took 2-4 hours to reach proposed definitions of ATC, and demonstrated variability in metabolic response. Further refinement of the model is indicated to improve reproducibility and clinical relevance. This may be achieved by titrating volume loss to variables shown to correlate with ATC (such as lactate or base deficit) rather than target volume or pressure, ensuring that the required degree of tissue hypoxia is consistently achieved.

Conclusion

This study presents an ovine model of coagulopathy in response to poly-trauma and hemorrhage. The model demonstrates changes in INR, aPTT and ROTEM parameters that are consistent with current clinical definitions of ATC. The degree of coagulopathy was closely correlated with the degree of shock as quantified by arterial lactate, supoorting the role of hypoperfusion in the development of ATC. The hypothesized role of the aPC pathway and endothelial glycocalyx in ATC was also supported, with coagulopathy

strongly correlated with protein C depletion and markers of endothelial glycocalyx shedding. With further refinement this model may facilitate mechanistic evaluation of hypothesized mechanisms, and enable evaluation of novel therapeutic strategies that may improve outcomes for trauma patients.

Acknowledgements

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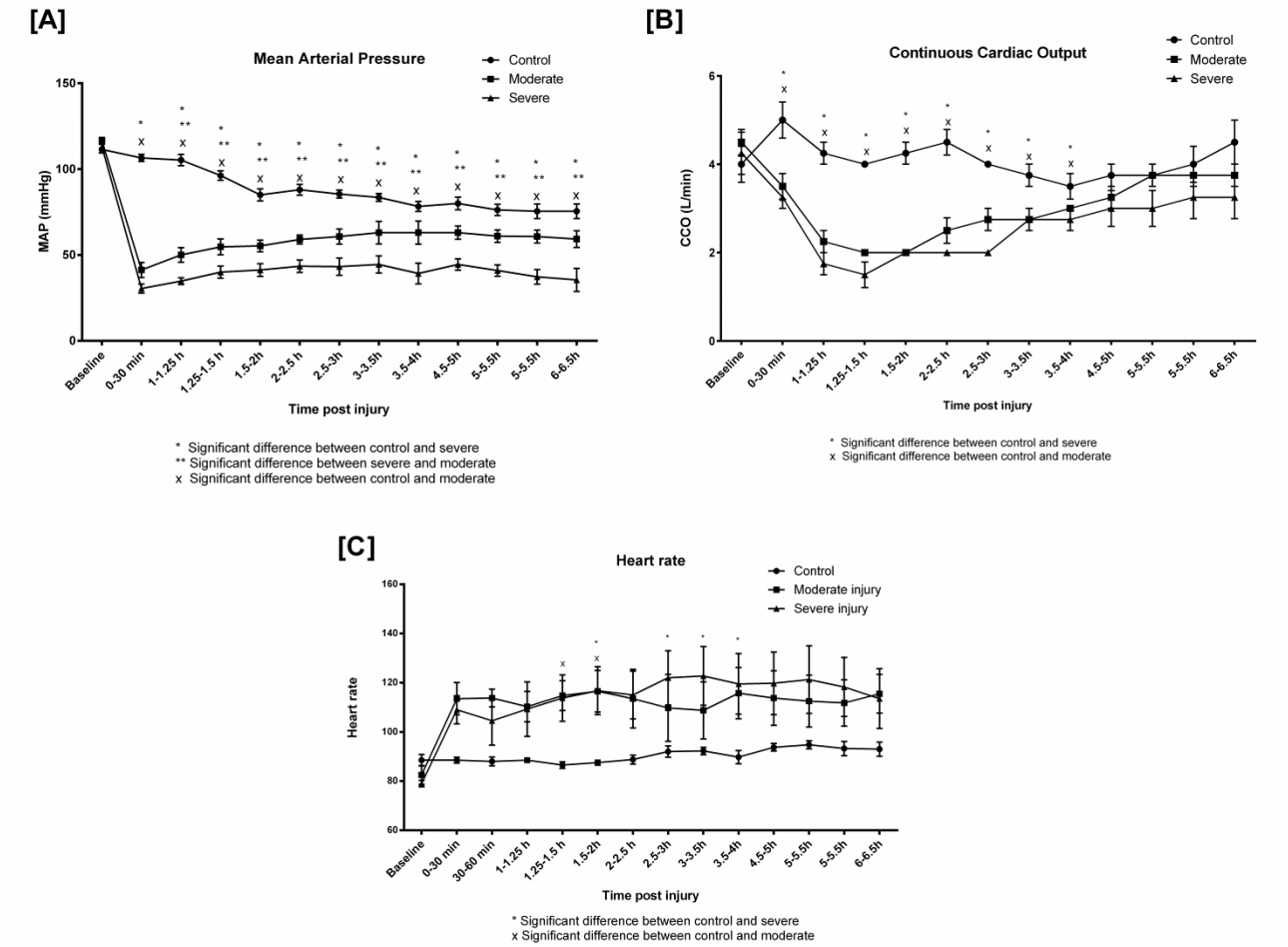

Figure 1. Hemodynamic changes following trauma and hemorrhage. [A] A significant reduction in mean arterial pressure (MAP) from baseline was evident in both trauma groups immediately post injury and maintained throughout the monitoring period. [B] A significant reduction in combined cardiac output (CCO) from baseline was evident in both trauma groups immediately post injury, with a return to baseline levels at 4 hours. [C] A significant elevation in heart rate was evident in both trauma groups from 1.5 hours post injury

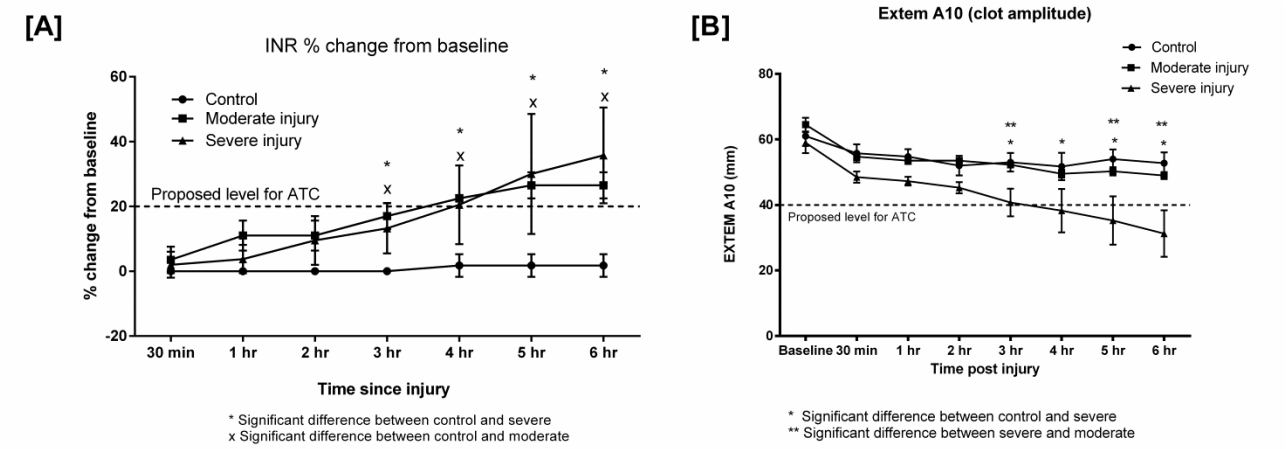

Figure 2. Coagulation changes following trauma and hemorrhage. [A] An increase in INR was evident in the moderate trauma (p=0.002) and severe trauma (p=0.008) groups compared to the control group, with a 20% increase from baseline evident at 4 hours. [B] A reduction in EXTEM A10 was evident in the severe trauma group (p=0.032) from 3 hours post injury.

Table 1. Hematology and metabolic parameters at baseline and time points following trauma and hemorrhage. Values are presented as mean±standard deviation

Variable	Baseline	Post splenic ligation	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Hb (g/L)									
Control	8.85±0.40	10.93±0.47	10.75±0.58	10.60±0.50	10.45±0.49	10.20±0.65	9.85±0.79	9.55±0.89	9.18±0.74
Moderate	8.38±0.30	9.95±0.27	8.38±0.28^{x}	8.10±0.22^{x}	7.90±0.14^{x}	7.40±0.01^{x}	6.98±0.10^{x}	6.70±0.08^{x}	6.58±0.05^{x}
Severe	7.56±0.76*	8.97±0.85	6.75±0.86*	6.43±0.66*	6.00±0.78*	5.66±0.86*	5.35±0.78*	5.20±1.12*	5.05±0.99*
Platelets (10^9/L)									
Control	456.3±90.61	(-)	373.5±52.25	356.5±57.38	341.0±69.60	337.5±54.79	328.8±66.17	319.0±65.67	282.0±70.11
Moderate	444.5±89.08		346.8±56.46	347.3±61.36	306.3±74.44	299.0±81.78	300.0±86.48	283.5±66.35	282.0±59.23
Severe	358.0±72.42		238.5±39.01*	229.8±46.54*	211.8±62.59*	206.0±69.78*	198.5±74.83*	205.3±84.31*	187.3±82.10*
MULTIPLATE ADP (U)									
Control	59.0±20.2	(-)	52.5±21.0	47.8±23.4	40.0±9.8	44.5±15.8	43.5±13.8	52.5±29.3	71.5±20.8
Moderate	65.0±21.6		54.5±22.0	59.0±28.0	57.8±6.4	57.8±29.8	67.3±29.0	81.0±26.9	65.3±28.8
Severe	40.5±15.0		32.5±6.4	40.0±2.2	42.3±18.8	47.0±14.6	39.0±13.8	40.0±12.8	45.8±21.5
MULTIPLATE Collagenase (U)									
Control	44.5±11.8	(-)	38.0±12.2	30.8±5.0	29.3±7.1	29.0±12.4	36.0±23.9	28.0±8.2	32.5±18.5
Moderate	36.0±15.4		26.0±13.8	29.0±27.4	44.5±11.4	35.3±15.4	34.8±15.2	40.0±19.5	38.5±23.5
Severe	30.3±9.2		27.3±1.6	26.5±9.9	25.3±15.4	23.0±7.6	34.5±3.2	22.3±7.9	17.8±4.5
Lactate(mmol/L)									
Control	1.23±0.38	0.58±0.28	0.58±0.20	0.53±0.20	0.53±0.16	0.55±0.20	0.50±0.14	0.55±0.14	0.55±0.09
Moderate	1.58±0.56	0.70±0.08	1.80±0.72	1.88±0.74	1.80±0.80	1.63±0.48	1.75±0.91	1.80±1.02	1.90±0.82
Severe	1.48±0.26	0.68±0.20	3.50±1.08	4.15±1.39*	4.68±1.93*	4.70±2.80*	5.58±4.10*	6.13±5.08*	6.73±5.30*
Base Excess (mmol/L)									
Control	2.28±2.00	5.68±1.90	6.20±2.02	7.46±1.86	7.43±2.02	7.13±2.06	7.35±1.95	7.45±2.14	7.58±2.62
Moderate	1.15±0.66	2.97±1.96	3.08±2.14	3.53±2.96	5.53±2.90	5.10±2.86	4.08±2.28	4.15±1.62	4.10±2.80
Severe	4.65±1.85	5.13±1.30	1.40±3.52	1.23±3.52*	1.03±5.68*	3.10±4.63	3.75±1.94	1.80±1.83*	1.60±1.37*
pH									
Control	7.37±0.04	7.41±0.02	7.41±0.02	7.43±0.02	7.42±0.04	7.42±0.02	7.42±0.02	7.43±0.04	7.43±0.04
Moderate	7.37±0.02	7.40±0.02	7.39±0.02	7.39±0.04	7.40±0.04	7.39±0.04	7.40±0.04	7.39±0.02	7.39±0.04
Severe	7.49±0.04	7.45±0.04	7.40±0.08	7.37±0.12	7.37±0.10	7.36±0.04	7.38±0.04	7.36±0.04	7.38±0.06
Bicarbonate (mmol/L)									
Control	26.7±2.46	28.6±2.24	29.7±2.16	30.2±2.40	31.7±2.20	31.6±3.02	31.3±2.34	31.3±2.30	31.5±2.66
Moderate	25.6±0.84	28.1±0.64	27.2±2.02	27.7±2.68	29.6±2.72	29.3±2.62	28.1±2.30	28.1±1.68	28.1±2.74
Severe	31.4±5.72	29.6±2.48	29.3±1.58	26.1±3.92	25.8±6.82*	25.6±3.70*	27.0±1.78	26.4±1.76	28.2±3.84

Hb = hemoglobin, Multiplate = induced platelet aggregation, (-) = not assessed, * = significant difference between control and severe trauma groups $p<0.05$. x = significant difference between control and moderate trauma groups $p<0.05$

Table 2. Coagulation function at baseline and at time points following trauma and hemorrhage. Values are presented as mean ± standard deviation

Variable	Baseline	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
aPTT (s)								
Control	24.25±1.50	23.50±1.92	23.25±1.89	24.00±2.00	24.00±2.00	23.75±2.88	24.25±2.50	24.75±2.50
Moderate	23.50±2.88	28.00±2.44	30.75±4.50^{x}	32.00±4.97^{x}	32.00±4.97^{x}	34.75±2.50^{x}	35.00±2.16^{x}	34.50±2.88^{x}
Severe	24.75±1.50	32.50±4.20*	33.00±2.58*	34.25±1.71*	34.25±1.71*	39.25±4.99*	42.00±8.16*	44.50±6.56*
INR								
Control	1.43±0.06	1.43±0.06	1.43±0.06	1.43±0.06	1.45±0.06	1.45±0.06	1.45±0.06	1.45±0.06
Moderate	1.33±0.06	1.38±0.10	1.48±0.06	1.48±0.06	1.55±0.06	1.63±0.06	1.65±0.06^{x}	1.65±0.06^{x}
Severe	1.33±0.10	1.35±0.06	1.38±0.10	1.43±0.06	1.48±0.10	1.60±0.20	1.73±0.32*	1.80±0.28*
Fibrinogen (g/L)								
Control	1.65±0.20	1.38±0.20	1.35±0.12	1.43±0.18	1.35±0.18	1.33±0.22	1.30±0.18	1.28±0.22
Moderate	1.78±0.18	1.18±0.18	1.18±0.10	1.08±0.06	1.10±0.02	1.10±0.02	1.10±0.02	1.03±0.06
Severe	1.88±0.18	1.48±0.22	1.28±0.24	1.18±0.24	1.13±0.22	0.98±0.20*	0.93±0.28*	0.85±0.20*
EXTEM								
A10 (mm)								
Control	61.00±6.06	55.75±4.44	54.75±4.58	52.00±5.94	53.00±5.72	51.75±8.34	54.00±6.80	52.75±6.60
Moderate	64.50±4.20	54.75±2.22	53.50±2.08	53.50±1.00	52.25±1.50	49.50±2.38	50.25±2.50	49.00±2.16
Severe	59.00±2.70	48.50±3.42	47.25±2.62	45.25±2.40	40.75±8.42*	38.25±13.2*	35.25±14.72*	31.25±14.2*
α angle (^{0})								
Control	71.67±1.92	66.00±17.0	65.00±6.40	66.00±5.90	67.67±8.98	69.33±5.00	70.33±6.66	70.67±5.56
Moderate	72.00±4.00	69.00±6.58	65.50±6.66	67.50±5.92	71.00±8.86	63.25±8.26	65.25±7.28	63.50±6.86
Severe	76.25±2.36	69.50±6.02	68.50±7.14	66.00±9.06	60.25±14.2	55.75±22.10	52.25±24.88	47.50±25.90*
CL_{30} (%)								
Control	100	100	100	100	100	100	100	100
Moderate	100	100	100	100	100	100	100	100
Severe	100	100	100	100	100	100	100	100
INTEM								
A10 (mm)								
Control	60.75±5.32	54.00±5.56	53.25±6.90	53.75±6.40	53.25±6.02	53.25±6.76	53.50±6.06	53.50±6.06
Moderate	64.75±3.20	56.00±2.22	56.50±2.38	56.25±1.70	54.00±1.42	54.25±2.62	53.00±0.82	53.00±0.82
Severe	58.25±5.56	53.00±2.82	50.75±2.50	50.00±4.24	46.75±4.58	44.00±8.52	41.75±10.88*	41.75±10.88*
α angle (^{0})								
Control	76.00±1.82	68.25±5.04	71.25±7.22	70.75±2.98	73.25±2.88	71.00±3.74	71.25±3.20	68.00±5.72
Moderate	73.25±3.50	70.25±2.98	71.25±2.98	72.75±1.70	70.00±3.16	69.25±2.06	69.00±2.44	68.50±5.74
Severe	78.50±0.58	73.25±4.37	72.50±3.32	70.25±2.88	66.25±7.98	62.50±13.53	55.50±19.06*	54.75±19.66*

CL_{30} (%)								
Control	100	100	100	100	100	100	100	100
Moderate	100	100	100	100	100	100	100	100
Severe	100	100	100	100	100	100	100	100
FIBTEM								
A10 (mm)								
Control	16.50±2.8	13.25±2.76	13.75±2.62	13.75±1.70	13.50±1.74	13.25±1.26	14.00±1.30	13.25±2.98
Moderate	18.00±3.82	13.75±2.22	13.25±1.90	12.75±2.50	12.00±2.58	11.50±3.32	11.75±2.06	11.75±1.26
Severe	21.00±1.42	16.50±2.08	15.50±2.08	14.50±2.64	14.25±4.28	12.50±3.70	11.50±4.20	11.75±352
α angle (^{o})								
Control	76.75±2.06	71.50±6.14	73.25±3.86	74.25±2.76	73.75±4.04	73.25±3.40	76.00±1.42	75.50±3.32
Moderate	78.00±4.54	72.50±4.50	73.00±2.54	69.75±7.64	67.25±14.94	68.00±7.88	68.75±6.94	67.75±5.68
Severe	79.75±0.96	75.75±3.68	74.75±4.72	73.00±4.32	69.00±10.24	69.75±7.28	65.75±13.82	65.50±15.02
CL_{30} (%)								
Control	100	100	100	100	100	100	100	100
Moderate	100	100	100	100	100	100	100	100
Severe	100	100	100	100	100	100	100	100

aPTT = activated partial thromboplastin time, INR = international normalised ratio A10 = clot amplitude at 10 minutes, CL_{30}= clot lysis index at 30 minutes,
* = significant difference between control and severe trauma groups $p<0.05$. x = significant difference between control and moderate trauma groups $p<0.05$

Table 3. Coagulation protease and endothelial glycocalyx markers at baseline and time points following trauma and hemorrhage. Values are presented as mean ± standard deviation

Variable	Baseline	30 min	1 hr	3 hr	5 hr
Factor VIII (U/ml)					
Control	12.05±1.44	11.2±0.58	10.26±1.22	10.70±2.04	10.17±1.96
Severe	10.38±0.52	7.92±1.34*	7.57±1.24*	6.33±0.32*	5.46±1.26*
Factor V (U/ml)					
Control	3.89±0.50	4.56±2.86	3.82±1.34	3.11±0.58	3.34±1.18
Severe	4.80±2.20	2.37±0.46	2.47±0.58	2.29±0.48§	1.79±0.88§
Protein C (mg/ml)					
Control	0.51±0.08	0.48±0.08	0.46±0.06	0.47±0.06	0.47±0.04
Severe	0.55±0.06	0.36±0.04*	0.32±0.06*	0.28±0.06*	0.22±0.06*
aPC (pg/ml)					
Control	278.3±35.6	(-)	257.3±29.2	317.8±15.8	323.3±37.6
Severe	283.8±139.8		462.0±158.4	589.3±203.6*	706.3±199.4*
PAI-1(U/ml)					
Control	7.32±0.22	6.98±0.30	6.99±0.54	7.04±0.54	7.34±0.64
Severe	6.84±0.70	5.96±0.14*	5.68±0.38*	4.69±0.38*	4.59±0.42*
sTM (ng/ml)					
Control	0.89±0.12	(-)	0.91±0.14	0.93±0.12	1.00±0.12
Severe	1.04±0.04		1.61±0.12*	1.63±0.16*	1.88±0.32*
D-dimer (ng/ml)					
Control	205.25±8.62	204.50±14.24	209.00±9.02	213.50±5.98	208.75±8.54
Severe	205.25±9.28	217.75±6.86	219.75±9.04	212.00±12.14	221.25±3.40
Syndecan-1(ng/ml)					
Control	10.00±0.82	(-)	11.25±0.50	10.75±0.96	12.00±0.82
Severe	10.00±0.96		14.25±0.96*	18.25±1.90*	20.50±2.64*
Hyaluranon (□g/ml)					
Control	0.29±0.16	0.47±0.32	0.55±0.30	0.38±0.34	0.43±0.38
Severe	0.13±0.04	1.07±0.34	1.94±0.44	2.32±1.04*	2.04±2.54*
Adrenaline (nmol/L)					
Control	0.65±0.30	0.35±0.06	0.20±0.12	0.25±0.14	0.23±0.18
Severe	0.88±0.46	0.20±0.08	0.28±0.18	0.35±0.26	0.48±0.28
Noradrenaline (nmol/L)					
Control	6.30±1.46	3.43±3.00	1.63±0.56	1.83±0.54	1.25±0.26
Severe	3.85±0.14	1.48±0.74	1.15±0.14	1.90±1.08	1.65±1.30

aPC = activated protein C, PAI-1 = plasminogen activator inhibitor-1, sTM = soluble thrombomodulin, (-) = not assessed, * = significant difference between control and severe groups ($p<0.05$), § = significant difference from baseline.

Table 4. Pearson correlation coefficients for coagulation function parameters, coagulation protease levels, endothelial glycocalyx markers and platelet values in the severe trauma group.

Parameter	Lactate	aPC	Protein C	F VIII	F V	PAI-1	sTM	Syndecan-1	Hyaluranon	Platelet count	Fibrinogen
EXTEM A10	-0.86****	-0.32	0.72***	0.78****	0.71****	0.49**	-0.64**	-0.89****	-0.93****	0.81****	0.78****
aPTT	0.70****	0.57*	-0.69***	-0.83****	-0.75****	(-)	(-)	0.91****	(-)	(-)	(-)
Protein C	-0.54*	-0.59*	(-)	0.78****	0.71***	0.79***	-0.89****	(-)	(-)	(-)	(-)
aPC	0.31	(-)	-0.59*	-0.57*	-0.25	-0.72**	0.62**	0.63**	(-)	(-)	(-)
Lactate	(-)	0.31	-0.54*	(-)	(-)	(-)	0.79***	0.62*	0.59*	(-)	-0.58***
PAI-1	(-)	-0.72**	0.79***	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0.73**
INTEM a angle	(-)	-0.29	0.66**	(-)	(-)	(-)	-0.69**	(-)	(-)	0.66****	0.72****

aPTT = activated partial thromboplastin time, aPC = activated protein C, PAI-1 = plasminogen activator inhibitor-1, F VIII = factor VIII, F V = Factor V, sTM = soluble thrombomodulin (-) = correlation not assessed. * = $p<0.05$, ** = $p<0.01$, ***=$p<0.001$, ****=$p<0.0001$

4.3 Discussion of accepted manuscript

At the commencement of this thesis the ability of sheep to demonstrate coagulopathy in response to a combination of trauma and haemorrhage had not been described in any available publications. The primary aim of the laboratory work performed in this thesis was therefore to design and develop a survivable ovine model of trauma and haemorrhage that demonstrated altered coagulation function in response to these insults. The observational assessment of proposed pathophysiological mechanisms was a secondary aim, and was undertaken using correlation analysis to facilitate identification of pathways, processes or therapeutics that may benefit from mechanistic evaluation in future studies.

The type of tissue trauma and degree of haemorrhage was designed using information obtained from the systematic review described in Chapter 3. As the response of the sheep to such insults was unknown the severity of trauma was increased in a step-wise fashion during the model development process. This was primarily to improve the ethical acceptability of the proposed project, as a significant degree of animal mortality would have precluded the model development studies from continuing. A 6 hour monitoring period was included for extended assessment of changes in coagulation function, as this information would facilitate future model refinement. All animals included in the study were from the same herd of origin and demonstrated no statistically significant differences in baseline physiological parameters.

To facilitate evaluation of ATC in this model attempts were made to minimise the impact of the physiological derangements of haemodilution, hypothermia and acidosis. Temperature was monitored throughout and normothermia maintained with the use of heating mats when required. A degree of endogenous haemodilution secondary to fluid shifts from extravascular sources was expected in the severe trauma groups secondary to hypovolaemia. However this was not compounded by iatrogenic haemodilution as the trauma animals received no fluid resuscitation, with crystalloid administration post injury minimised to 10ml/hr in all animals. The trauma animals also failed to demonstrate a significant acidosis despite the observed rise in lactate levels, most likely due to the development of a co-existing metabolic alkalosis of unknown cause.

Sheep demonstrate a significantly lower resting haemoglobin (Hb) in comparison to humans (59) which results from physiological differences in splenic function. The ovine

spleen stores up to 26% of the circulating red cell mass and boosts circulating Hb levels during periods of physiological stress (236), a function that is lacking in the human spleen (235). All animals in this thesis underwent a splenectomy to attenuate this difference in auto-transfusive capacity. The splenectomy was preceded by intra-splenic administration of adrenaline to induce splenic contracture (237) to increase the pre-injury Hb levels to the equivalent of human values. Splenectomy is a realistic component of an injury scenario and was considered for inclusion during the injury phase in this model without induced splenic contracture. However in the sheep this would also remove a variable amount of circulating red cell mass, which may increase variability in response and influence the reproducibility of the model. As a result it was included as part of the instrumentation phase in this thesis.

The severe trauma animals in this thesis demonstrated changes in INR, aPTT and EXTEM A10 at 2-4 hours post injury that were consistent with current definitions of ATC. This thesis therefore achieved the aim of producing an ovine model of trauma haemorrhage that demonstrated clinically relevant coagulation changes. However in human trauma patients ATC is usually evident within 30 minutes of injury (22, 89). The proposed model therefore has limitations, such as the time taken to achieve a clinically relevant coagulopathy, that indicate the need for ongoing refinement. The overall findings, limitations and future research directions for this model are discussed in further detail in Chapter 5.

CHAPTER 5: DISCUSSION

5.1 Key Findings

The ultimate aim of this thesis was achieved, with the creation of the first ovine model of trauma and haemorrhage to achieve proposed definitions of ATC. The design of the model was based upon a reproducible and survivable tissue injury that was equivalent to an ISS of 25, and was combined with fixed volume haemorrhage as it was deemed more clinically significant and facilitated assessment of the haemodynamic responses to hypovolaemia. The human dimensions of the sheep enabled more detailed observational assessment of proposed pathophysiological contributors than has previously been published in any other experimental model. This has provided further evidence of the importance of tissue hypoperfusion in the development of ATC, with the onset of coagulopathy strongly correlated with arterial lactate levels. It has also further implicated the protein C pathway as a key component of pathophysiology, and provided additional evidence of the possible contribution of endothelial glycocalyx degradation to ATC development. The findings of this thesis have therefore added to the understanding of ATC pathogenesis, and produced an experimental model that with ongoing refinement may be useful for further investigation of ATC and its management. These findings are discussed in further detail in the following sections.

5.1.1 The suitability of sheep as a model of acute traumatic coagulopathy

The success of ovine models in biomedical research is well recognised, with established models of critical illness and haemostatic diseases widely published. This success combined with established similarities with humans in coagulation parameters (58, 59), and haemodynamic, microcirculatory and immunological function (75-77) made them an attractive species in which to develop a model of ATC. A validated ovine model of blood transfusion also exists and demonstrates good comparability with humans in terms of red cell preparation, lifespan and storage lesions (244). Future evaluation of different haemostatic resuscitation regimens in an ovine model of ATC may therefore improve the translatability of results and better inform subsequent human studies.

The model described in this thesis adds to the list of successful ovine models, as it is the first described ovine model of trauma and haemorrhage to achieve ATC as defined by INR, aPTT and EXTEM A10. It is also the first large animal of trauma and haemorrhage to achieve proposed clinical definitions of ATC without associated mortality in the injury

group(s). The aim of creating a survivable tissue injury that was equivalent to an ISS of 25 was therefore achieved. This may have been facilitated by the established similarities between human and ovine coagulation function (58, 59). The severity of tissue injury and degree of haemorrhage required to produce a clinically relevant response in sheep may have been less than in other species that demonstrate established differences with human coagulation function.

The similarities in size between humans and sheep allowed standard human monitoring equipment to be utilised, which provided detailed information on the haemodynamic responses to trauma and haemorrhage in this model. Collection of multiple blood samples throughout the study period was also possible due to animal size, allowing more in depth evaluation of proposed pathophysiological mechanisms. As a result the model utilised in this thesis is the only animal model of ATC to have evaluated aspects of all major hypothesised pathophysiological contributors (table 4).

Table 4. *Hypothesised pathophysiological mechanisms evaluated in animal models of trauma and haemorrhage that achieve clinical definitions of ATC*.

Author	Animal Species	Coagulation findings	Pathophysiological features evaluated
van Zyl et al	Sheep	20% increase in INR aPTT > 34s EXTEM A10 < 40mm	*Protein C pathway* (aPC, FV, FVIII, PAI-1, sTM) *Glycocalyx* (syndecan-1, hyaluranon) *Fibrinolysis* (D-dimer, FIBTEM, Fibrinogen, Histology) *Platelet function* (Multiplate induced aggregation testing) *Hypoperfusion* (Lactate, Base deficit)
Duan et al	Pig	20% increase in INR/PT Prolonged R value	*Fibrinolysis* (ATIII, Fibrinogen, D-dimer *Hypoperfusion* (Lactate, Base deficit)
Darlington et al	Rat	PT > 18s aPTT > 24s Decreased MCF	*Hypoperfusion* (Lactate, Base deficit) *Fibrinolysis* (Fibrinogen, FIBTEM)
Frith et al	Rat	PTr = 1.3	*Hypoperfusion* (Lactate, Base deficit)
Chesebro et al	Mouse	aPTT > 34s	*Hypoperfusion* (Lactate, Base deficit) *Protein C pathway* (Monoclonal protein C antibodies)

5.1.2 Tissue hypoperfusion, blood lactate levels, base deficit and coagulopathy.

Epidemiological work strongly suggests that the development of ATC requires a combination of tissue injury and tissue hypoperfusion., with coagulopathy most likely to occur if an ISS >15 is combined with a base deficit of > 6mmol/L (29). Retrospective studies and experimental models support this finding, with coagulopathy not developing in the presence of normal base deficit, regardless of the injury severity score (29, 37, 92, 93).

The findings of this thesis further support the role of hypoperfusion in the development of coagulopathy. Tissue hypoperfusion causes inadequate oxygen delivery to the tissues, forcing the onset of anaerobic metabolism that leads to the accumulation of lactic acid (245). The severe trauma group in this thesis demonstrated a strong correlation between coagulopathy as defined by both aPTT and EXTEM A10 and arterial lactate levels. However the severe trauma animals did not develop an associated base deficit or acidosis despite the significant rise in arterial lactate. Base deficit is mechanistically linked to arterial lactate levels, and is often used as a surrogate marker for the accumulation of lactic acid as it has been shown to correlate with arterial lactate in animal models of shock (246). However clinical studies evaluating trauma and critical illness have shown poor correlation between base deficit and arterial lactate levels (245, 247, 248). Clinical studies have also shown arterial lactate to be a strong predictor of outcomes such as mortality and need for resuscitation in trauma (249, 250), and arterial lactate was a better predictor of haemorrhage severity than base deficit in an LD50 model of penetrating trauma (251). There are no publications that assess the sensitivity of arterial lactate as a predictor of ATC. However in the trauma animals in this thesis arterial lactate appeared to be a better predictor of coagulopathy than base deficit.

All animals in this thesis (including controls) demonstrated a significant increase in bicarbonate levels during the experimental period, suggesting the development of a metabolic alkalosis. It is likely that a co-existing metabolic alkalosis in the trauma sheep blunted the changes in base deficit expected to accompany the observed rise in blood lactate levels. The cause of the metabolic alkalosis in the animals used in this thesis is unknown. The development of a metabolic alkalosis has been seen previously in sheep using alternate forms of total intravenous anaesthesia (252, 253), and while there are no published reports associated with alfaxalone it is possible it may be secondary to the anaesthetic agent. Sheep do also demonstrate significant salivary and ruminal losses during anaesthesia (254). Ruminant foregut physiology would suggest that these losses

should be pH neutral or high in bicarbonate, which should not trigger the development of a metabolic alkalosis (254, 255). However no analysis of the gastric losses from the animals used in this thesis was undertaken. Movement of acid from the abomasum towards the rumen may have occurred, resulting in acid losses and triggering the development of alkalosis. Further investigation of these changes should be considered in future studies.

5.1.3 Changes in coagulation function

Coagulation function within this model was assessed using both traditional plasma based assays and viscoelastic assays of coagulation function. This was in keeping with the multiple proposed definitions of ATC, and the traditional and contemporary assessment of coagulation dysfunction in the trauma patient (22, 23, 29-34). ATC was originally characterised using PT, aPTT and/or INR, and these tests remain the current standard for establishing a definitive diagnosis of coagulopathy. Studies suggest PT/INR to be more sensitive indicators of coagulopathy in trauma (256, 257), whilst aPTT appears to be more specific (98). However these traditional assays were originally developed to identify specific factor deficiencies within the extrinsic or intrinsic pathways of coagulation resulting from heritable coagulopathies or anticoagulant therapy (258). They have been shown to correlate poorly with bleeding risk in patients undergoing elective surgical procedures (259), and as they require 30-60 minutes for processing they may not accurately reflect the rapidly evolving coagulation status of the trauma patient (33). This has led to the increasing use of viscoelastic testing in the trauma setting, as EXTEM A5 and A10 and FIBTEM A5 have all shown to be sensitive predictors of coagulopathy (30, 32, 34).

Assessment of INR within this model was performed using both formal laboratory testing and a point of care (POC) measuring device (Coaguchek XS System, Roche Diagnostics, NSW, Australia). The accuracy of POC devices in the evaluation of traumatic coagulopathy in the emergency setting has been assessed in retrospective and prospective studies (154-158). In the majority of these studies the results from the POC device were well correlated with formal laboratory tests, allowed rapid availability of results and appeared cost effective (154-157). The rationale behind the use of POC devices in this thesis was twofold. Firstly it was to provide some immediate feedback with regards to INR levels during the experimental period, which assisted with the model development process. Secondly it was to evaluate the correlation between POC and laboratory INR values in this experimental model. The results from the POC device were well correlated with the results from the formal laboratory tests within this model (figure 7). This is

consistent with findings in human studies and suggests that POC INR devices may play a role in the early identification of the coagulopathic trauma patient, particularly in the pre-hospital setting or in facilities that are unable to access viscoelastic point of care testing.

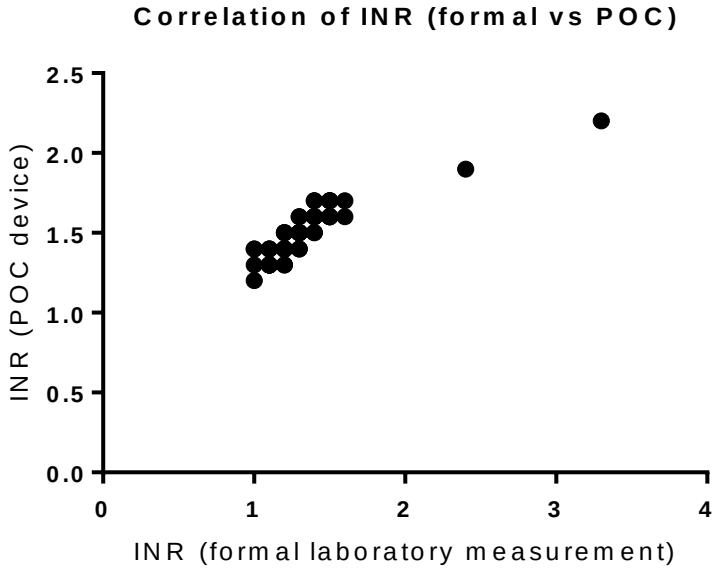

Figure 7. *A strong correlation between laboratory and point of care INR measurements was evident in the severe trauma group (R=0.8746, p<0.0001)*

The severe trauma animals in this thesis demonstrated significant changes in ROTEM EXTEM A10 values compared to controls, with proposed definitions of ATC achieved at 3 hours post injury (30). Changes in EXTEM A10, EXTEM A5 and FIBTEM A5 values on ROTEM have been shown to be strongly predictive of massive transfusion in trauma in observational studies (30, 32, 34). FIBTEM A5 appeared the most sensitive predictive measure in one study, leading to suggestions it should be used in combination with EXTEM A10 or A5 in the detection and resuscitation of the coagulopathic trauma patient. (34). However it is recognised that significant fibrinolysis appears to develop in a subset of patients with ATC (39, 96, 111, 112), and the sensitivity of FIBTEM A5 is likely to be highlighted in this group. In this thesis there were no significant changes in any FIBTEM parameters or D-dimer levels, suggesting fibrinolysis did not make a significant contribution to the coagulopathy observed in the proposed model. The presence of fibrinolysis in trauma has been associated with a higher ISS and base deficit (89, 111), and it is possible that the aim of developing a survivable injury in this thesis inhibited the development of a model that demonstrated significant fibrinolysis. It has also been demonstrated that FIBTEM assays are relatively insensitive to fibrinolysis, detecting only 10% of patients with plasmin-antiplasmin complex confirmed fibrinolysis (112). Additional

testing is therefore warranted in future studies to better characterise fibrinolysis and its contribution to ATC.

5.1.4 The contribution of the protein C pathway.

A strong association between coagulopathy and the protein C pathway was evident in the model described in this thesis. The degree of coagulopathy was correlated with an increase in sTM and aPC, and a decrease in protein C, factor V, factor VIII and PAI-1 levels in the severe trauma animals. This suggests protein C activation, inhibition of the coagulation cascade and the onset of global anti-coagulation (37-39). These findings support existing evidence suggesting that activation of the protein C pathway may play a significant role in the development of ATC (37-40).

Protein C is a systemic antiocoagulant that is proteolytically converted to an active form by thrombin bound concurrently to TM and the EPCR (16, 37). Once activated it proceeds to inactivate factors Va and VIIIa and deplete PAI-1 (16, 260). Activation of the protein C pathway serves two primary protective functions. The first is inhibition of systemic thrombosis following tissue injury through the binding of thrombin to constitutively expressed thrombomodulin on undamaged endothelial cells (17, 260). The second is inhibition of local thrombosis during periods of hypoperfusion via the increased expression of thrombomodulin on activated endothelial cells (16, 17).

The proposed trigger for the pathological activation of protein C in ATC is the widespread release of thrombomodulin from endothelial cells in response to tissue hypoperfusion (37). Clinical evidence has demonstrated that systemic hypoperfusion is associated with an increase in sTM levels (37, 89). This was supported by the findings of this thesis, with the severe trauma animals demonstrating a significant rise in sTM levels that was correlated with arterial lactate measurements. Furthermore the rise in sTM observed in the severe trauma animals was correlated with protein C depletion, aPC rise and degree of coagulopathy. There is some debate in the literature regarding the relationship between sTM and endothelial bound thrombomodulin (eTM), with studies variously suggesting that sTM may reflect overall TM activity (261, 262), inhibit the action of eTM (263, 264) or act simply as a marker of endothelial injury (265). However the association between sTM and aPC levels in this thesis suggests that sTM levels reflect overall thrombomodulin activity.

Widespread activation of protein C impairs thrombin utilisation, resulting in impaired clot formation. ROTEM EXTEM A10 provides a quantitative assessment of mean clot firmness (164, 165), and the reduction in EXTEM A10 in this ovine model provides evidence of impaired clot formation. The correlation between aPC and EXTEM A10 values in this thesis suggests that aPC may have contributed to this finding. Protein C activation also results in consumption of PAI-1, which may cause uninhibited tPA mediated conversion of plasminogen to plasma. In this thesis a decrease in PAI-1 was correlated with the increase in sTM and aPC levels, suggesting that activation of protein C may have triggered PAI-1 inhibition. However there was no evidence of overt fibrinolysis in this thesis, which is consistent with suggestions that aPC is unable to PAI-1 to the extent required to produce a clinically relevant fibrinolysis (41).

5.1.5 The contribution of the endothelial glycocalyx

The endothelial glycocalyx is a layer of cell bound proteoglycans and glycoproteins that acts as a key regulator of vascular permeability, cell adhesion and inflammation (130, 266, 267). The major constituents of the endothelial glycocalyx are syndecan, hyaluronic acid, chondroitin sulphate and heparin sulphate (266, 267). Disruption of the endothelial glycocalyx leads to endothelial cell activation, platelet adhesion and the expression of anticoagulant or pro-fibrinolytic proteins (130, 268, 269).

An increase in syndecan-1 has been associated with coagulopathy and mortality in trauma patients, suggesting that degradation of the endothelial glycocalx may contribute to the development of ATC (45, 46, 132). The findings of this thesis further support this hypothesis, with both syndecan-1 and hyaluronon correlated with the degree of coagulopathy as measured by EXTEM A10 and aPTT. However the mechanism by which endothelial glycocalyx shedding contributes to coagulopathy is still unclear. A volume of plasma containing heparin like substances is also held within the glycocalyx, and release of this following degradation may have direct anticoagulant effects from endogenous heparinisation (46). An alternative hypothesis is that shedding triggers increased fibrinolysis and thrombin generation, which in the presence of increased thrombomodulin from endothelial cell damage may trigger protein C activation and global anticoagulation (45, 135). Further work to better evaluate the mechanistic contribution of the microvasculature in ATC is therefore indicated.

Traumatic injury and haemorrhagic shock result in tissue ischaemia, activation of the inflammatory system and stimulation of the neuro-humoral axis with a subsequent catecholamine surge (45, 131-134). Changes in the endothelial glycocalyx in trauma have predominately been attributed to this catecholamine surge, with sympathetic blockade effective in preventing coagulopathy and endothelial glycocalyx shedding in rodent models of haemorrhagic shock (241, 242). However this finding was not supported in this thesis. No elevation in plasma catecholamine levels was evident in either trauma group, and there was no association between glycocalyx degradation and catecholamine levels evident on correlation analysis. Instead the increase in both syndecan-1 and hyaluronon was strongly correlated with arterial lactate levels. Ischaemia has been shown to be a significant trigger for endothelial glycocalyx degradation in patients undergoing vascular surgery (134). The findings of this thesis suggest that ischaemia secondary to hypoperfusion may have been the trigger for endothelial glycocalyx degradation in the severe trauma animals. It is therefore possible that tissue hypoperfusion rather than catecholamine excess is the trigger for endothelial glycocalyx degradation in trauma patients.

5.1.6 Altered platelet function did not contribute to coagulopathy in this model

The cell based model of haemostasis recognises the fundamental role of platelets in the balanced assembly of a stable fibrin clot (14). Trauma patients demonstrate decreased responsiveness to platelet aggregation testing, leading to speculation that dysfunction of platelet activation and adhesion pathways may contribute to ATC (47, 48, 140). However differences in platelet function may exist between platelets at the site of injury and the circulating platelet pool (270), with further evaluation required to ascertain if circulating platelets truly reflect active platelet function.

In this thesis the severe trauma animals developed significant thrombocytopaenia immediately post injury which persisted throughout the monitoring period. This is consistent with human findings in ATC, in which minor reductions in the admission platelet count are predictive of mortality (138, 139). Thrombocytopaenia in the severe trauma animals was correlated with the reduction in EXTEM A10 and INTEM alpha angle, which suggests that thrombocytopaenia may have contributed to reductions in clot formation and clot strength. However there was no associated change in induced platelet aggregation parameters to suggest altered platelet function. Constitutional differences in platelet function may have contributed to this finding as the magnitude of ADP and collagen induced platelet aggregation in sheep is less than half that of humans (59). It is possible

that a significant alteration in platelet function did develop, however was unable to be detected by the tests utilised in this thesis.

5.1.7 Limitations of the model used in this thesis

Pre-clinical animal research will always have associated limitations, and the model used in this thesis is no different. The lack of catecholamine release by the trauma animals in this thesis suggests that the ethical necessities of sedation and anaesthesia may have significantly altered the compensatory physiological responses to injury (211), impacting on the overall response that was observed. In contrast to human trauma patients who simultaneously experience tissue injury and uncontrolled haemorrhage, this thesis applied standardised insults in a staggered fashion to control the degree of trauma and haemorrhage experienced by the animals. This non-pathophysiological sequence of events may have also influenced the overall response observed by further impacting on the compensatory response to injury in the trauma animals.

Sheep are genetically distinct from humans, and despite demonstrable similarities in coagulation function also have acknowledged differences in primary and secondary haemostasis that may impact the translatability of findings (58, 59). These differences include constitutively different concentrations of coagulation factors, with mean protein C activity that is 60% of human values and mean factor VIII activity that is three times those of humans (59) (table 5). Sheep also demonstrate significant differences in platelet response to induced aggregation testing. Ovine platelets have proven unresponsive to TRAP-6, arachidonic acid and risocetin agonists and show less than 50% of the human response to ADP and collagen agonists (59, 62) (table 5), which may make it difficult to accurately assess the contribution of platelet dysfunction to coagulopathy in this model.

Table 5. *Ovine ranges for selected coagulation parameters compared to human reference ranges.*

Coagulation test	**Sheep (SBL)** **Mean ± SD**	**Human** **Reference Range**
Protein C activity (%)	48.6±9.3	70-140
Factor VIII activity (%)	816.9±201.4	50-150
ADP induced platelet aggregation (U)	38±20	57-113
Collagen induced platelet aggregation (U)	41±19	72-125

ADP=adenosine diphosphate, SBL = Samm Border Leisceter. All values taken from Foley et al 2014 (54)

ATC develops within 30 minutes of injury in human patients (22, 37, 89). In this thesis a significant prolongation of aPTT was evident within 30 minutes of injury in the severe trauma animals, however proposed definitions of ATC were only achieved at 2-4 hours following injury. The severe trauma animals also demonstrated variability in haemostatic and metabolic responses to injury. One animal become noticeably more unwell than the other animals in the group, demonstrating a much higher rise in plasma lactate concentration and INR and greater reduction in EXTEM A10 (see appendix 7.1.1). The results reported for this study included this animal because power calculations required a minimum of 4 animals and it was felt 3 animals were insufficient to give valid quantitative results. However the impact of this animal on overall results was assessed by performing a basic secondary statistical analysis with this animal removed (see appendix 7.1.2). A statistically significant difference in EXTEM A10, aPTT and INR was maintained following removal of this animal, although a difference in the time point at which statistical significance was achieved was evident. Variability of response can be expected in any *in vivo* study and is taken into account when performing power calculations, particularly given the need to minimise the number of animals required for statistical validity (271). However the degree of variability in the severe trauma animals in this study was unexpected. Both the variability in response and the time taken to achieve clinical definitions of ATC indicate that further refinement of the model is required to improve clinical relevance and reproducibility for future studies.

5.2 Future research direction

5.2.1 Refining the proposed model

The overarching aim of this thesis was the development of an ovine model of trauma and haemorrhage that demonstrated coagulopathy consistent with current definitions of ATC. This aim was achieved with the severe trauma group demonstrating an increase in aPTT to 34.25 ± 0.85 s at 2 hours post injury, decrease in EXTEM A10 to 40.75 ± 4.21mm at 3 hours post injury and increase in INR by 20.50 ± 6.08% at 4 hours post injury (23, 29, 30). This confirms our supposition that sheep may be a suitable species for the development of a large animal model of ATC. However as mentioned in the previous section ATC is usually evident in human patients within 30 minutes of injury. The time taken for a clinically relevant coagulopathy to develop in the model used in this thesis, and the variability of response evident in the severe trauma animals indicates that ongoing model refinement is required to improve reproducibility and clinical relevance.

Fixed volume haemorrhage was used in this model as it was felt it more closely mimicked the clinical situation, and facilitated the assessment of the haemodynamic responses to hypovolaemia (215). However haemorrhage of 30% blood volume in this model failed to produce a significant change in pH or base deficit which suggests that an increase in volume loss is indicated. However estimating the degree by which volume loss needs to be increased is difficult given the variability in response that was observed in the severe trauma animals. As the volume removed was based upon body weight the pre-experimental state of the animal influenced the degree of blood loss. All animals used in this study were fasted for 12 hours prior to surgical intervention, which resulted in loss of 2.4±1.9kg of body weight from pre-fasting measurements. Although the difference in fasting weight loss is small, it is possible that it may have contributed to the variability in physiologic and metabolic response that was observed. Fixed pressure haemorrhage could be used as an alternative as it would allow the degree and duration of hypotension to be controlled (215). However the need for repeated blood withdrawal or volume administration to maintain target pressure is not reflective of the clinical situation and introduces potential confounders. Maintaining pressure with crystalloids may result in iatrogenic haemodilution (272) whilst administration of anti-coagulated blood may cause iatrogenic anti-coagulation (67).

Epidemiological work in human patients has shown that ATC is most likely to develop if an ISS of 25 or greater is combined with a minimum base deficit of 6mmol/L (29). The model used in this thesis was designed to simulate an ISS of 25 based upon this work. As a result it may be worthwhile targeting haemorrhage to a variable predictive of ATC, rather than a target blood pressure or volume as these have not proven to predict the onset of coagulopathy. While a significant rise in base deficit was not evident in this thesis the severe trauma animals did demonstrate a significant rise in blood lactate levels, which have proven to be a sensitive predictor of mortality and need for resuscitation in clinical studies (249, 250). Qualitative evaluation of data from this study and other published experimental models suggests that animals that develop a lactate value of 4mmol/L or more following trauma and haemorrhage were more likely to develop a clinically relevant coagulopathy (table 6) (29, 40, 65, 67). Therefore it may be more appropriate to target haemorrhage to a minimum blood lactate level of 4mmol/L, rather than a fixed volume based upon body weight or a fixed pressure. This may improve the reproducibility of the proposed model, whilst also accelerating the time point at which a clinically relevant coagulopathy becomes evident.

Table 6. *Lactate values and coagulation changes in published animal trauma and haemorrhage models.*

Authors	Mean lactate values post trauma and haemorrhage	Clinically relevant coagulopathy	Coagulation findings
van Zyl et al	1.88±0.37mmol/L at 1hr (Moderate)	Borderline	aPTT 34.75±2.50s (4hrs) INR ↑22.5±1.0% (4hrs)
	4.15±0.70mmol/L at 1hr (Severe)	Yes	aPTT 34.25±1.71s (2hrs) INR ↑22.5±6.0% (4hrs) EXTEM A10 40.75±8.42 (3hrs)
Duan et al (67)	4.98±1.4mmol/L at 10min	Yes	INR ↑ 23±0.7% (40 min)
Darlington et al (65)	5.6±0.4mmol/L at 40min	Yes	PT 21.6±0.3s (1hr)
Frith et al (29)	8.6±2.0mmol/L	Yes	PT 23.5±2.4s
Chesebro et al (40)	10.7±3.6mmol/L at 1hr	Yes	aPTT 35.3±3.1s (1hr)
Mulier et al (202)	2.0±1.3mmol/L at 45min	No	Increased CFT on TEG.

5.2.2 Further investigation of proposed pathophysiological mechanisms

This thesis evaluated changes in platelet function, markers of endothelial glycocalyx degradation, components of the protein C pathway and fibrinolytic function that accompanied the onset of coagulopathy in the severe trauma animals. As a result it encompassed aspects of all major hypothesised pathophysiological mechanisms (270). However as this thesis was primarily aimed at model development the examination of proposed pathophysiological mechanisms was purely observational in nature. Calculated correlation coefficients examined the relationships between coagulopathy and various biomarkers, further supporting hypothesised contributions of the endothelial glycocalyx and protein C pathway. However correlation does not equate to causation and further mechanistic studies are indicated to better elucidate the mechanisms underlying the development of ATC.

The ability to evaluate isolated pathways within the coagulation system has been demonstrated in a murine model of ATC, in which the administration of activated protein C antibodies stopped the development of coagulopathy (40). However more detailed investigation in rodent models has been limited by animal size and established differences in coagulation function (58, 224). Refinement of the model used in this thesis may facilitate

mechanistic evaluation of ATC on a larger scale via the interference or manipulation of isolated pathways. The development of ovine antibodies to various aspects of the protein C pathway would facilitate a more detailed assessment of the contribution of various components of the pathway to ATC. Assessment of plasmin-antiplasmin complexes would better reflect the degree of fibrinolysis in the model (112), and manipulation of PAI-1 and tPA may improve understanding of the mechanism underlying fibrinolysis. A better understanding of the microcirculation and endothelial glycocalyx may be elucidated through the use of electron microscopy, organ microdialysis and sidestream dark field camera application (136, 273, 274). The role of endogenous heparinisation could also be better assessed via the use of anti-Xa assays (275). Immunohistochemistry (IHC) to investigate the tissue expression of biomarkers such as thrombomodulin in addition to measuring circulating levels will also contribute to improved understanding, particularly given the controversies regarding the action of sTM. This was considered in this thesis; however no commercially available ovine thrombomodulin antibodies suitable for IHC were able to be located. Cross reactivity with human (thrombomodulin/BDCA antibody, R&D systems), bovine (thrombomodulin antibody, Biorbyt) and caprine (thrombomodulin antibody, Enzo Life Sciences Inc) antibodies was assessed, with none found. Thrombomodulin IHC would therefore require the development of ovine specific antibodies which was beyond the scope of this thesis.

The evaluation of additional pathophysiological theories is also indicated in future work. Coagulation dysfunction triggers a de novo systemic inflammatory response, which may contribute to the increased incidence of sepsis and multiorgan failure in trauma patients (50, 276). ATC has been associated with increased cytokine expression (most notably TNF-α and IL-1) and complement activation via the release of mitochondrial damage-associated molecular patterns (DAMPs) (276). Immune activation may therefore aggravate tissue damage and amplify haemostatic activation. In addition trauma patients have demonstrated an increase in platelet derived microparticles (PMPs) that were associated with increased blood product requirements and mortality (148, 149). These PMPs are thought to also have pro-inflammatory effects and may play an important role in linking coagulation and inflammation; however the precise contribution to ATC is still undetermined. Methodological evaluation of inflammatory markers and PMPs in a controlled fashion using an *in vivo* model may help elucidate the contribution of these additional factors to ATC.

5.2.3 Investigation of proposed therapeutic strategies.

Haemostatic or 'damage control' resuscitation regimens form the current core therapeutic approach to patients with traumatic injury. It is perceived that these regimens restore circulating volume and reverse coagulopathy, resulting in improved patient outcomes (51, 52, 81, 82). However a recent prospective study demonstrated worsening haemostatic function in trauma patients despite the use of haemostatic resuscitation, suggesting factors other than correction of coagulopathy may contribute to the survival benefits associated with these regimens (80).

The composition of haemostatic resuscitation varies between countries and institutions (84-86), reflecting a lack of understanding of the benefits and risks of individual blood components in trauma. There is some evidence to suggest that fresh frozen plasma (FFP) may have a protective and restorative effect on the endothelial glycocalyx (137, 178), a property that has not been described with fibrinogen concentrate or cryoprecipitate. This may make FFP an attractive option for inclusion in resuscitation regimens given the proposed contribution of the glycocalyx to ATC. However a systematic review evaluating the comparative clinical effectiveness of FFP and fibrinogen demonstrated no significant difference in outcomes (277). Refinement of the model proposed by this thesis would allow more detailed evaluation of the effects of FFP and other resuscitation components on the endothelial glycocalyx and haemostatic response. Differing resuscitation regimens could also be compared, which may better inform subsequent human studies.

5.3 Concluding statement

Pre-clinical animal research is a necessary adjunct for improving the understanding and management of ATC given the acknowledged limitations of human research in the emergency setting. Creating a clinically relevant model of ATC that facilitates translation of results to humans is difficult given the complexity of the condition and known limitations of animal research. This thesis describes the development of the first ovine model of trauma and haemorrhage to demonstrate coagulation changes consistent with current definitions of ATC. The degree of coagulopathy was closely correlated with the degree of shock as quantified by arterial lactate levels, further supporting the importance of tissue hypoperfusion in the development of ATC. Observational assessment of proposed pathophysiological mechanisms within the model demonstrated a strong correlation between coagulopathy and activation of the protein C pathway, which supports

suggestions that protein C activation may play a central role in the development of ATC. Coagulopathy was also strongly correlated with an increase in markers of endothelial glycocalyx shedding, providing further evidence that the endothelial glycocalyx may contribute to the development of coagulopathy in trauma. However there was no evidence of fibrinolysis and no significant changes in induced platelet aggregation testing to suggest that fibrinolysis or platelet dysfunction made a significant contribution to coagulopathy in this thesis.

Clinical definitions of ATC were evident 2-4 hours following injury in this thesis and the severe trauma animals did demonstrate variability in the metabolic and haemostatic response to trauma. This indicates the need for ongoing refinement of the proposed model in order to improve reproducibility and clinical relevance. Refinement may facilitate mechanistic evaluation of proposed pathophysiological mechanisms, which may improve understanding of the condition and identify novel therapeutic targets. Refining the proposed model would also provide a platform for the evaluation of targeted and novel haemostatic interventions in a whole biological system, which may reduce exposure to blood products and help improve outcomes for trauma patients.

CHAPTER 6: REFERENCE LIST

1. Centre for Disease Control and Prevention. Web-based Injury Statistics Query and Reporting System (WISQARS) [Internet] 2012 [cited 2016 June 29]. Available from: **http://www.cdc.gov/injury/wisqars/pdf/leading_causes_of_death_by_age_group_2012-a.pdf**.
2. Chiara O, Cimbanassi S. Organized trauma care: does volume matter and do trauma centers save lives? *Curr Opin Crit Care*. 2003;9(6):510-514.
3. Sauaia A, Moore FA, Moore EE, Moser KS, Brennan R, Read RA, et al. Epidemiology of trauma deaths: a reassessment. *J Trauma*. 1995;38(2):185-193.
4. World Health Organisation. The Global Burden of Disease: 2004 Update. Geneva, Switzerland: World Health Organisation, 2004.
5. Evans JA, van Wessem KJ, McDougall D, Lee KA, Lyons T, Balogh ZJ. Epidemiology of traumatic deaths: comprehensive population-based assessment.*World J Surg*. 2010;34(1):158-163.
6. Demetriades D, Murray J, Charalambides K, Alo K, Velmahos G, Rhee P, et al. Trauma fatalities: time and location of hospital deaths. *J Am Coll Surg*. 2004;198(1):20-26.
7. Martin M, Oh J, Currier H, Tai N, Beekley A, Eckert M, et al. An analysis of in-hospital deaths at a modern combat support hospital. *J Trauma*. 2009;66(4 Suppl):S51-60
8. Acosta JA, Yang JC, Winchell RJ, Simons RK, Fortlage DA, Hollingsworth-Fridlund P, et al. Lethal injuries and time to death in a level I trauma center. *J Am Col Surg* 1998;186(5):528-533.
9. Becker BF, Heindl B, Kupatt C, Zahler S. Endothelial function and hemostasis. *Z Kardiol.* 2000;89(3):160-167.
10. Jackson SP, Nesbitt WS, Westein E. Dynamics of platelet thrombus formation. *J Thromb Haemost*. 2009;7(Suppl 1):17-20.
11. Rivera J, Lozano Mí L, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*. 2009;94(5):700-711.
12. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting *Science* 1964;145(3638):1310-1312.
13. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier *Nature*.1964;202:498-499.

14. Hoffman M, Monroe DM. A cell-based model of hemostasis. *Thromb Haemost*. 2001;85(6):958-965.
15. Kubier A, O'Brien M. Endogenous anticoagulants. *Top Companion Anim Med*. 2012;27(2):81-87.
16. Esmon CT. The protein C pathway. *Chest.* 2003;124(3 Suppl):26s-32s.
17. Esmon CT. The normal role of Activated Protein C in maintaining homeostasis and its relevance to critical illness. *Crit Care*. 2001;5(Suppl 2):S7-s12.
18. Broze GJ, Jr. Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu Rev Med*. 1995;46:103-112.
19. Hoyt DB, Bulger EM, Knudson MM, Morris J, Ierardi R, Sugerman HJ, et al. Death in the operating room: an analysis of a multi-center experience. *J Trauma* 1994;37(3):426-432.
20. Armand R, Hess J. Treating coagulopathy in trauma patients. *Transfus Med Rev*. 2003;17:223-231.
21. Cap A, Hunt BJ. The pathogenesis of traumatic coagulopathy. *Anaesthesia*. 2015;70(Suppl 1):96-101
22. Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. *J Trauma* 2003;54(6):1127-1130.
23. MacLeod J, Lynn M, McKenney M, Cohn S, Murtha M. Early coagulopathy predicts mortality in trauma. *J Trauma* 2003;55(1):39-44.
24. Maegele M, Lefering R, Yucel N, Tjardes T, Rixen D, Paffrath T, et al. Early coagulopathy in multiple injury: an analysis from the German Trauma Registry on 8724 patients. *Injury.* 2007;38(3):298-304.
25. Niles SE, McLaughlin DF, Perkins JG, Wade CE, Li Y, Spinella PC, et al. Increased mortality associated with the early coagulopathy of trauma in combat casualties. The *J Trauma*. 2008;64(6):1459-1463
26. Hess JR, Lindell AL, Stansbury LG, Dutton RP, Scalea TM. The prevalence of abnormal results of conventional coagulation tests on admission to a trauma center. *Transfusion*. 2009;49(1):34-39.
27. O'Shaughnessy DF, Atterbury C, Bolton Maggs P, Murphy M, Thomas D, Yates S, et al. Guidelines for the use of fresh-frozen plasma, cryoprecipitate and cryosupernatant. *Br J Haematol*. 2004;126(1):11-28.
28. Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on

Perioperative Blood Transfusion and Adjuvant Therapies. *Anesthesiology*. 2006;105(1):198-208.

29. Frith D, Goslings JC, Gaarder C, Maegele M, Cohen MJ, Allard S, et al. Definition and drivers of acute traumatic coagulopathy: clinical and experimental investigations. *J Thromb Haemost* 2010;8(9):1919-1925
30. Woolley T, Midwinter M, Spencer P, Watts S, Doran C, Kirkman E. Utility of ROTEM values of clot strength, A5 and A10, in predicting final assessment of coagulation status in severely injured battle patients. *Injury*. 2013;44(5):593-599.
31. Carroll RC, Craft RM, Langdon RJ, Clanton CR, Snider CC, Wellons DD, et al. Early evaluation of acute traumatic coagulopathy by thrombelastography. *Transl Res* 2009;154(1):34-39.
32. Davenport R, Manson J, De'Ath H, Platton S, Coates A, Allard S, et al. Functional definition and characterization of acute traumatic coagulopathy. *Crit Care Med* 2011;39(12):2652-2658.
33. Holcomb JB, Minei KM, Scerbo ML, Radwan ZA, Wade CE, Kozar RA, et al. Admission rapid thrombelastography can replace conventional coagulation tests in the emergency department: experience with 1974 consecutive trauma patients. *Ann Surg* 2012;256(3):476-486.
34. Hagemo JS, Christiaans SC, Stanworth SJ, Brohi K, Johansson PI, Goslings JC, et al. Detection of acute traumatic coagulopathy and massive transfusion requirements by means of rotational thromboelastometry: an international prospective validation study. *Crit Care* 2015;19:97.
35. Rugeri L, Levrat A, David JS, Delecroix E, Floccard B, Gros A, et al. Diagnosis of early coagulation abnormalities in trauma patients by rotation thrombelastography. *J Thromb Haemost* 2007;5(2):289-295.
36. Hunt H, Stanworth S, Curry N, Woolley T, Cooper C, Ukoumunne O, et al. Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) for trauma induced coagulopathy in adult trauma patients with bleeding. *Cochrane Database Syst Rev* 2015(2):Cd010438.
37. Brohi K, Cohen MJ, Ganter MT, Matthay MA, Mackersie RC, Pittet JF. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? *Ann Surg*. 2007;245(5):812-818.
38. Cohen MJ, Call M, Nelson M, Calfee CS, Esmon CT, Brohi K, et al. Critical role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. *Ann Surg*. 2012;255(2):379-385.

39. Cohen MJ, Kutcher M, Redick B, Nelson M, Call M, Knudson MM, et al. Clinical and mechanistic drivers of acute traumatic coagulopathy. *J Trauma Acute Care Surg*. 2013;75(1 Suppl 1):S40-47.
40. Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, et al. Increase in activated protein C mediates acute traumatic coagulopathy in mice. *Shock.* 2009;32(6):659-665.
41. Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. *J Thromb Haemost*. 2005;3(1):35-45.
42. Gando S. Acute coagulopathy of trauma shock and coagulopathy of trauma: a rebuttal. You are now going down the wrong path. *J Trauma*. 2009;67(2):381-383.
43. Gando S, Wada H, Kim HK, Kurosawa S, Nielsen JD, Thachil J, et al. Comparison of disseminated intravascular coagulation in trauma with coagulopathy of trauma/acute coagulopathy of trauma-shock. *J Thromb Haemost*. 2012;10(12):2593-2595.
44. Campbell J, Meledeo M, Cap A. Comparative response of platelet fV and plasma fV to activated protein C and relevance to a model of acute traumatic coagulopathy. *Plos One.* 2014;9(6):e99181.
45. Johansson PI, Stensballe J, Rasmussen IS, Ostrowski SR. A high admission syndecan-1 level, a marker of endothelial glycocalyx degredation, is associated with inflammation, protein C depletion, fibrinolysis and increased mortality in trauma patients. *Ann Surg*. 2011;254:194-200.
46. Ostrowski SR, Johansson PI. Endothelial glycocalyx degradation induces endogenous heparinization in patients with severe injury and early traumatic coagulopathy. *J Trauma Acute Care Surg*. 2012;73(1):60-66.
47. Wohlauer MV, Moore EE, Thomas S, Sauaia A, Evans E, Harr J, et al. Early platelet dysfunction: an unrecognized role in the acute coagulopathy of trauma. *J Am Coll Surg*. 2012;214(5):739-746.
48. Solomon C, Traintinger S, Ziegler B, Hanke A, Rahe-Meyer N, Voelckel W, et al. Platelet function following trauma. A multiple electrode aggregometry study. *Thromb Haemost*. 2011;106(2):322-330.
49. Dunne JR, Malone DL, Tracy JK, Napolitano CM. Allogenic blood transfusion in the first 24 hours after trauma is associated with increased systemic inflammatory response syndrome (SIRS) and death. *Surg Infect (Larchmt)*. 2004;5:395-404.
50. Esmon CT. The interactions between inflammation and coagulation. *Br J Haematol.* 2005;131:417-430.

51. Doran CM, Doran CA, Woolley T, Carter A, Male K, Midwinter MJ, et al. Targeted resuscitation improves coagulation and outcome. *J Trauma Acute Care Surg* 2012;72(4):835-843.
52. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, et al. Damage control resuscitation: Directly addressing the early coagulopathy of trauma. *J Trauma*. 2007;62:307-310.
53. Howard BM, Daley AT, Cohen MJ. Prohemostatic interventions in trauma: resuscitation-associated coagulopathy, acute traumatic coagulopathy, hemostatic resuscitation, and other hemostatic interventions. *Semin Thromb Hemost* 2012;38(3):250-258
54. Reichel CA, Lerchenberger M, Uhl B, Rehberg M, Berberich N, Zahler S, et al. Plasmin Inhibitors Prevent Leukocyte Accumulation and Remodeling Events in the Postischemic Microvasculature. *PLoS ONE*. 2011;6(2):e17229.
55. Chen TT, Jiandong L, Wang G, Jiang SL, Li LB, Gao CQ. Combined treatment of ulinastatin and tranexamic acid provides beneficial effects by inhibiting inflammatory and fibrinolytic response in patients undergoing heart valve replacement surgery. *Heart Surg Forum*. 2013;16(1):E38-47.
56. Curry N, Hopewell S, Doree C, Hyde C, Brohi K, Stanworth S. The acute management of trauma hemorrhage: a systematic review of randomized controlled trials. *Crit Care*. 2011;15(2):R92.
57. Ho AM, Dion PW, Yeung JH, Holcomb JB et al. Prevalence of survivor bias in observational studies on fresh frozen plasma : erythrocyte ratios in trauma requiring massive transfusion. *Anaesthesiology*. 2012;116(3):716-728.
58. Siller-Matula JM, Plasenzotti R, Spiel A, Quehenberger P, Jilma B. Interspecies differences in coagulation profile. *Thromb Haemost*. 2008;100(3):397-404
59. Foley SR SC, Simonova G, Spanevello MM, Bird RJ, Semple JW, Jackson DE, Schibler A, Fraser JF, Fung YL. A comprehensive study of ovine haemostasis to assess suitability to model human coagulation. *Thromb Res* 2014;134:468-473.
60. Velik-Salchner C, Schnurer C, Fries D, Mussigang PR, Moser PL, Streif W et al. Normal values for thromboelastography (ROTEM) and selected coagulation parameters in porcine blood. *Thromb Res*. 2006;117:597-602.
61. Karges HE, Funk KA, Ronneberger H. Activity of coagulation and fibrinolysis parameters in animals. *Arzneimittel-Forschung*. 1994;44(6):793-797.

62. Baumgarten A, Wilhelmi M, Kalbantner K, Ganter M, Mischke R. Measurement of platelet aggregation in ovine blood using a new impedance aggregometer.*Vet Clin Pathol* 2010;39(2):149-156.
63. van Zyl N, Reade MC, Fraser JF. Experimental Animal Models of Traumatic Coagulopathy: A Systematic Review. *Shock.* 2015.
64. Frith D, Cohen MJ, Brohi K. Animal models of trauma-induced coagulopathy. *Thromb Res*. 2012;129(5):551-556.
65. Darlington DN, Craig T, Gonzales MD, Schwacha MG, Cap AP, Dubick MA. Acute coagulopathy of trauma in the rat. *Shock*. 2013;39(5):440-446.
66. Swindle MM, Makin A, Herron AJ, Clubb FJ Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol.* 2012;49(2):344-356.
67. Duan K, Yu W, Lin Z, Tan S, Bai X, Xu L, et al. A time course study of acute traumatic coagulopathy prior to resuscitation: from hypercoagulation to hypocoagulation caused by hypoperfusion? *Transfus Apher Sci* 2014;50(3):399-406.
68. El Mays TY, Choudhury P, Leigh R, Koumoundouros E, Van der Velden J, Shrestha G, et al. Nebulized perflubron and carbon dioxide rapidly dilate constricted airways in an ovine model of allergic asthma. *Respir Res*. 2014;15(1):98.
69. Scheerlinck JP, Snibson KJ, Bowles VM, Sutton P. Biomedical applications of sheep models: from asthma to vaccines. *Trends Biotechnol*. 2008;26(5):259-266.
70. Bindl R, Oheim R, Pogoda P, Beil FT, Gruchenberg K, Reitmaier S, et al. Metaphyseal fracture healing in a sheep model of low turnover osteoporosis induced by hypothalamic-pituitary disconnection (HPD). *Orthop Res*. 2013;31(11):1851-1857.
71. Enkhbaatar P, Murakami K, Traber LD, Cox R, Parkinson JF, Westphal M, et al. The inhibition of inducible nitric oxide synthase in ovine sepsis model. *Shock*. 2006;25(5):522-527.
72. Tung JP, Fung YL, Nataatmadja M, Colebourne KI, Esmaeel HM, Wilson K, et al. A novel in vivo ovine model of transfusion-related acute lung injury (TRALI). *Vox Sang* 2011;100(2):219-230.
73. Kim WG, Lee BH, Seo JW. Light and electron microscopic analyses for ischaemia-reperfusion lung injury in an ovine cardiopulmonary bypass model. *Perfusion.* 2001;16(3):207-214.
74. Lange M, Hamahata A, Enkhbaatar P, Esechie A, Connelly R, Nakano Y, et al. Assessment of vascular permeability in an ovine model of acute lung injury and

pneumonia-induced Pseudomonas aeruginosa sepsis. *Crit Care Med*. 2008;36(4):1284-1289.

75. Hein WR, Griebel PJ. A road less travelled: large animal models in immunological research. *Nat Rev Immnol*. 2003;3(1):79-84.
76. Kitchen H. Sheep as animal models in biomedical research. *J Am Vet Med Assoc* 1977;170(6):615-619.
77. Maybauer MO, Maybauer DM, Fraser JF, Szabo C, Westphal M, Kiss L, et al. Recombinant human activated protein C attenuates cardiovascular and microcirculatory dysfunction in acute lung injury and septic shock. *Crit Care*. 2010;14(6):R217.
78. Geeraedts L, Kaasjager H, van Vugt A, Frolke J. Exsanguination in trauma: A review of diagnostics and treatment options. *Injury*. 2009;40(1):11-20.
79. Paffrath T, Wafaisade A, Lefering R, Simanski C, Bouillon B, Spanholtz T, et al. Venous thromboembolism after severe trauma: incidence, risk factors and outcome. *Injury.* 2010;41(1):97-101.
80. Khan S, Davenport R, Raza I, Glasgow S, De'Ath HD, Johansson PI, et al. Damage control resuscitation using blood component therapy in standard doses has a limited effect on coagulopathy during trauma hemorrhage. *Intensive Care Med*. 2015;41(2):239-247.
81. Johansson PI ,Stensballe J. Haemostatic resuscitation for massive bleeding: the paradigm of plasma and platelets - a review of the current literature. *Transfusion*. 2010;50:701-710.
82. Johansson PI, Stensballe J. Effect of Haemostatic Control Resuscitation on mortality in massively bleeding patients: a before and after study. *Vox Sang*. 2009;96(2):111-118.
83. Snyder CW, Weinberg JA, McGwin G, Jr., Melton SM, George RL, Reiff DA, et al. The relationship of blood product ratio to mortality: survival benefit or survival bias? *J Trauma*. 2009;66(2):358-362
84. Spoerke NJ, Van PY, Differding JA, Zink KA, Cho SD, Muller PJ, et al. Red blood cells accelerate the onset of clot formation in polytrauma and hemorrhagic shock. *J Trauma*. 2010;69(5):1054-1059
85. Zink KA, Sambasivan CN, Holcomb JB, Chisholm G, Schreiber MA. A high ratio of plasma and platelets to packed red blood cells in the first 6 hours of massive transfusion improves outcomes in a large multicenter study. *Am J Surg*. 2009;197(5):565-570

86. Davenport R, Curry N, Manson J, De'Ath H, Coates A, Rourke C, et al. Hemostatic effects of fresh frozen plasma may be maximal at red cell ratios of 1:2. *J Trauma*. 2011;70(1):90-95
87. Schochl H, Nienaber U, Hofer G, Voelckel W, Jambor C, Scharbert G, et al. Goal-directed coagulation management of major trauma patients using thromboelastometry (ROTEM)-guided administration of fibrinogen concentrate and prothrombin complex concentrate. *Crit Care*. 2010;14(2):R55.
88. Faller DV. Endothelial cell responses to hypoxic stress. *Clin Exp Pharmacol Physiol*. 1999;26(1):74-84.
89. Brohi K, Cohen MJ, Ganter MT, Schultz MJ, Levi M, Mackersie RC, et al. Acute coagulopathy of trauma: hypoperfusion induces systemic anticoagulation and hyperfibrinolysis. *J Trauma* 2008;64(5):1211-1217
90. Rutherford EJ, Morris JA, Jr., Reed GW, Hall KS. Base deficit stratifies mortality and determines therapy. *J Trauma* 1992;33(3):417-423.
91. Jansen JO, Scarpelini S, Pinto R, Tien HC, Callum J, Rizoli SB. Hypoperfusion in severely injured trauma patients is associated with reduced coagulation factor activity. *J Trauma*. 2011;71(5 Suppl 1):S435-440.
92. MacLeod JB, Winkler AM, McCoy CC, Hillyer CD, Shaz BH. Early trauma induced coagulopathy (ETIC): prevalence across the injury spectrum. *Injury*. 2014;45(5):910-915.
93. Sixta SL, Hatch QM, Matijevic N, Wade CE, Holcomb JB, Cotton BA. Mechanistic determinates of the acute coagulopathy of trauma (ACoT) in patients requiring emergency surgery. *Int J Burns Trauma*. 2012;2(3):158-166.
94. Hagemo JS, Stanworth S, Juffermans NP, Brohi K, Cohen M, Johansson PI, et al. Prevalence, predictors and outcome of hypofibrinogenaemia in trauma: a multicentre observational study. *Crit Care*. 2014;18(2):R52.
95. Ostrowski SR, Sorensen AM, Larsen CF, Johansson PI. Thrombelastography and biomarker profiles in acute coagulopathy of trauma: a prospective study. *Scand J Trauma Resusc Emerg Med*. 2011;19:64.
96. Cardenas JC, Matijevic N, Baer LA, Holcomb JB, Cotton BA, Wade CE. Elevated tissue plasminogen activator and reduced plasminogen activator inhibitor promote hyperfibrinolysis in trauma patients. *Shock*. 2014;41(6):514-521.
97. van Hinsbergh VW, Bertina RM, van Wijngaarden A, van Tilburg NH, Emeis JJ, Haverkate F. Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium. *Blood.* 1985;65(2):444-451.

98. Brohi K CM, Davenport RA. Acute coagulopathy of trauma: mechanism, identification and effect. *Curr Opin Crit Care*. 2007;13:680-685.
99. Kornblith LZ, Kutcher ME, Redick BJ, Calfee CS, Vilardi RF, Cohen MJ. Fibrinogen and platelet contributions to clot formation: implications for trauma resuscitation and thromboprophylaxis. *J Trauma Acute Care Surg*. 2014;76(2):255-256
100. Rourke C, Curry N, Khan S, Taylor R, Raza I, Davenport R, et al. Fibrinogen levels during trauma hemorrhage, response to replacement therapy, and association with patient outcomes. *J Thromb Haemost*. 2012;10(7):1342-1351.
101. Schochl H, Cotton B, Inaba K, Nienaber U, Fischer H, Voelckel W, et al. FIBTEM provides early prediction of massive transfusion in trauma. *Crit Care*. 2011;15(6):R265.
102. Inaba K, Karamanos E, Lustenberger T, Schochl H, Shulman I, Nelson J, et al. Impact of fibrinogen levels on outcomes after acute injury in patients requiring a massive transfusion. *J Am Coll Surg*. 2013;216(2):290-297.
103. Hagemo JS, Jorgensen J, Ostrowski SR, Holtan A, Gundersen Y, Johansson PI, Naess PA, Gaarder C. Changes in fibrinogen availability and utilization in an animal model of traumatic coagulopathy. *Scand J Trauma Resusc Emerg Med.*. 2013;21:56.
104. Stinger HK, Spinella PC, Perkins JG, Grathwohl KW, Salinas J, Martini WZ, et al. The ratio of fibrinogen to red cells transfused affects survival in casualties receiving massive transfusions at an army combat support hospital. *J Trauma* 2008;64(2 Suppl):S79-85.
105. Curry N, Rourke C, Davenport R, Beer S, Pankhurst L, Deary A, et al. Early cryoprecipitate for major haemorrhage in trauma: a randomised controlled feasibility trial. *Brit J Anaesth*. 2015;115(1):76-83..
106. National Blood Authority. Patient blood management guidelines: Module 3 - Medical. Australia 2012..
107. Winearls J. Fibrinogen Concentrate vs Cryoprecipitate in Traumatic Haemorrhage: A Pilot Randomised Controlled Trial 2016 [cited 2016 27 June 2016]. NCT02745041]. Available from: https://clinicaltrials.gov/ct2/show/NCT02745041.
108. Martini WZ. Fibrinogen metabolic responses to trauma. *Scand J Trauma Resusc Emerg Med*. 2009;17:2.
109. Sawamura A, Hayakawa M, Gando S, Kubota N, Sugano M, Wada T, et al. Disseminated intravascular coagulation with a fibrinolytic phenotype at an early phase of trauma predicts mortality. *Thromb Res*. 2009;124(5):608-613.

110. Davenport RA, Brohi K. Cause of trauma-induced coagulopathy. *Curr Opin Anaesthesiol.* 2015.
111. Cotton BA, Harvin JA, Kostousouv V, Minei KM, Radwan ZA, Schochl H, et al. Hyperfibrinolysis at admission is an uncommon but highly lethal event associated with shock and prehospital fluid administration. *J Trauma Acute Care Surg* 2012;73(2):365-370
112. Raza I, Davenport R, Rourke C, Platton S, Manson J, Spoors C, et al. The incidence and magnitude of fibrinolytic activation in trauma patients. *J Thromb Haemost* 2013;11(2):307-314.
113. Levrat A, Gros A, Rugeri L, Inaba K, Floccard B, Negrier C, et al. Evaluation of rotation thrombelastography for the diagnosis of hyperfibrinolysis in trauma patients. *Brit J Anaesth* 2008;100(6):792-797.
114. Ives C, Inaba K, Branco BC, Okoye O, Schochl H, Talving P, et al. Hyperfibrinolysis elicited via thromboelastography predicts mortality in trauma. *J Am Coll Surg* 2012;215(4):496-502.
115. Schochl H, Frietsch T, Pavelka M, Jambor C. Hyperfibrinolysis after major trauma: differential diagnosis of lysis patterns and prognostic value of thrombelastometry. *J Trauma* 2009;67(1):125-131.
116. Shakur H, Roberts I, Bautista R, Caballero J, Coats T, Dewan Y, et al. Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial. *Lancet*. 2010;376(9734):23-32.
117. Morrison JJ, Dubose JJ, Rasmussen TE, Midwinter MJ. Military Application of Tranexamic Acid in Trauma Emergency Resuscitation (MATTERs) Study. *Arch Surg.* 2012;147(2):113-119.
118. Chandler WL. Procoagulant activity in trauma patients. *Am J Clin Path*. 2010;134(1):90-96.
119. Brakenridge SC, Henley SS, Kashner TM, Golden RM, Paik DH, Phelan HA, et al. Comparing Clinical Predictors of Deep Venous Thrombosis vs. Pulmonary Embolus After Severe Injury: A New Paradigm for Post-Traumatic Venous Thromboembolism? *J Trauma Acute Care Surg*. 2013;74(5):1231-1238.
120. Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet JM, Tedgui A. Endothelial microparticles in diseases. *Cell Tissue Res*. 2009;335(1):143-151.
121. Holley AD, Reade MC. The 'procoagulopathy' of trauma: too much, too late? *Curr Opin Crit Care* 2013;19(6):578-586

122. Aird WC. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood*. 2003;101(10):3765-3777.
123. Harlan JM, Winn RK. Leukocyte-endothelial interactions: Clinical trials of anti-adhesion therapy. *Crit Care Med*. 2002;30(5):S214-S219.
124. Vincent JL, Sun Q, Dubois MJ. Clinical trials of immunomodulatory therapies in severe sepsis and septic shock. *Clin Infect Dis*. 2002;34(8):1084-1093.
125. Gando S, Wada H, Thachil J. Differentiating disseminated intravascular coagulation (DIC) with the fibrinolytic phenotype from coagulopathy of trauma and acute coagulopathy of trauma-shock (COT/ACOTS). *J Thromb Haemost* 2013;11(5):826-835.
126. Moore HB, Moore EE, Lawson PJ, Gonzalez E, Fragoso M, Morton AP, et al. Fibrinolysis shutdown phenotype masks changes in rodent coagulation in tissue injury versus hemorrhagic shock. *Surgery*. 2015;158(2):386-392.
127. Kushimoto S, Gando S, Saitoh D, Ogura H, Mayumi T, Koseki K, et al. Clinical course and outcome of disseminated intravascular coagulation diagnosed by Japanese Association for Acute Medicine criteria. Comparison between sepsis and trauma. *Thromb Haemost*. 2008;100(6):1099-1105.
128. Sawamura A, Hayakawa M, Gando S, Kubota N, Sugano M, Wada T, et al. Application of the Japanese Association for Acute Medicine disseminated intravascular coagulation diagnostic criteria for patients at an early phase of trauma. *Thromb Res* 2009;124(6):706-710.
129. Rizoli S, Nascimento B, Jr., Key N, Tien HC, Muraca S, Pinto R, et al. Disseminated intravascular coagulopathy in the first 24 hours after trauma: the association between ISTH score and anatomopathologic evidence. *J Trauma*. 2011;71(5 Suppl 1):S441-447.
130. Nieuwdorp M, Meuwese MC, Vink H, Hoekstra JB, Kastelein JJ, Stroes ES. The endothelial glycocalyx: a potential barrier between health and vascular disease.*Curr Opin Lipidol*. 2005;16(5):507-511.
131. Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR. High circulating adrenaline levels at admission predict increased mortality after trauma. *J Trauma Acute Care Surg* 2012;72(2):428-436.
132. Johansson PI, Ostrowski SR. Acute coagulopathy of trauma: balancing progressive catecholamine induced endothelial activation and damage by fluid phase anticoagulation. *Med Hypotheses*. 2010;75(6):564-567.

133. Becker BF, Jacob M, Leipert S, Salmon AH, Chappell D. Degradation of the endothelial glycocalyx in clinical settings: searching for the sheddases. *Br J Clin Pharmacol*. 2015;80(3):389-402.
134. Rehm M, Bruegger D, Christ F, Conzen P, Thiel M, Jacob M, et al. Shedding of the endothelial glycocalyx in patients undergoing major vascular surgery with global and regional ischemia. *Circulation*. 2007;116(17):1896-1906.
135. Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. *Diabetes*. 2006;55(2):480-486.
136. Reitsma S, Slaaf DW, Vink H, van Zandvoort M, oude Egbrink MGA. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Archiv*. 2007;454(3):345-359.
137. Kozar RA, Peng Z, Zhang R, Holcomb JB, Pati S, Park P, et al. Plasma restoration of endothelial glycocalyx in a rodent model of hemorrhagic shock. *Anesth Analg*. 2011;112(6):1289-1295.
138. Brown LM, Call MS, Margaret Knudson M, Cohen MJ, Holcomb JB, Wade CE, et al. A normal platelet count may not be enough: the impact of admission platelet count on mortality and transfusion in severely injured trauma patients. *J Trauma*. 2011;71(2 Suppl 3):S337-342.
139. Stansbury LG, Hess AS, Thompson K, Kramer B, Scalea TM, Hess JR. The clinical significance of platelet counts in the first 24 hours after severe injury. *Transfusion*. 2013;53(4):783-789.
140. Kutcher ME, Redick BJ, McCreery RC, Crane IM, Greenberg MD, Cachola LM, et al. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg*. 2012;73(1):13-19.
141. Holcomb JB, Tilley BC, Baraniuk S, Fox EE, Wade CE, Podbielski JM, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA* 2015;313(5):471-482.
142. Inaba K, Branco BC, Rhee P, Blackbourne LH, Holcomb JB, Spinella PC, et al. Impact of the duration of platelet storage in critically ill trauma patients. *J Trauma*. 2011;71(6):1766-1773.
143. Donahue DL, Beck J, Fritz B, Davis P, Sandoval-Cooper MJ, Thomas SG, et al. Early Platelet Dysfunction in a Rodent Model of Blunt Traumatic Brain Injury

Reflects the Acute Traumatic Coagulopathy Found in Humans. *J Neurotrauma* 2014;31(4):404-410.

144. Pareti FI, Capitanio A, Mannucci L, Ponticelli C, Mannucci PM. Acquired dysfunction due to the circulation of "exhausted" platelets. *Am J Med*. 1980;69(2):235-240.
145. Moore HB, Moore EE, Chapman MP, Gonzalez E, Slaughter AL, Morton AP, et al. Viscoelastic measurements of platelet function, not fibrinogen function, predicts sensitivity to tissue-type plasminogen activator in trauma patients. *J Thromb Haemost* 2015;13(10):1878-1887.
146. Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost*. 2009;101(3):439-451.
147. Morel N, Morel O, Petit L, Hugel B, Cochard JF, Freyssinet JM, et al. Generation of procoagulant microparticles in cerebrospinal fluid and peripheral blood after traumatic brain injury. *J Trauma*. 2008;64(3):698-704.
148. Windelov NA, Sorensen AM, Perner A, Wanscher M, Larsen CF, Ostrowski SR, et al. Platelet aggregation following trauma: a prospective study. *Blood Coagul Fibrinolysis*. 2014;25(1):67-73.
149. Matijevic N, Wang YW, Wade CE, Holcomb JB, Cotton BA, Schreiber MA, et al. Cellular microparticle and thrombogram phenotypes in the Prospective Observational Multicenter Major Trauma Transfusion (PROMMTT) study: correlation with coagulopathy. *Thromb Res*. 2014;134(3):652-658.
150. Park MS, Xue A, Spears GM, Halling TM, Ferrara MJ, Kuntz MM, et al. Thrombin generation and procoagulant microparticle profiles after acute trauma: A prospective cohort study. *J Trauma Acute Care Surg*. 2015;79(5):726-731.
151. Curry N, Raja A, Beavis J, Stanworth S, Harrison P. Levels of procoagulant microvesicles are elevated after traumatic injury and platelet microvesicles are negatively correlated with mortality. *J Extracell Vesicles*. 2014;3.
152. Dzik WH. Predicting hemorrhage using preoperative coagulation screening assays. *Curr Hematol Rep*. 2004;3(5):324-330.
153. Johansson PI, Stissing T, Bochsen L, Ostrowski SR. Thrombelastography and tromboelastometry in assessing coagulopathy in trauma. *Scand J Trauma Resusc Emerg Med*. 2009;17:45.
154. Beynon C, Erk AG, Potzy A, Mohr S, Popp E. Point of care coagulometry in prehospital emergency care: an observational study. *Scand J Trauma Resusc Emerg Med*. 2015;23.

155. Goodman MD, Makley AT, Hanseman DJ, Pritts TA, Robinson BR. All the bang without the bucks: Defining essential point-of-care testing for traumatic coagulopathy. *J Trauma Acute Care Surg* 2015;79(1):117-124.
156. Cotte J, D'Aanda E, Chauvin V, Kaiser E, Meaudre E. Point-of-Care Coagulation Testing for Trauma Patients in a Military Setting: A Prospective Study. *J Spec Oper Med* 2013;13(4):59-62.
157. David JS, Levrat A, Inaba K, Macabeo C, Rugeri L, Fontaine O, et al. Utility of a point-of-care device for rapid determination of prothrombin time in trauma patients: a preliminary study. *J Trauma Acute Care Surg*. 2012;72(3):703-707.
158. Mitra B, O'Reilly G, Collecutt M, Cameron PA, Phillips L, Davis A. Prospective comparison of point-of-care international normalised ratio measurement versus plasma international normalised ratio for acute traumatic coagulopathy. *Emerg Med Australas*. 2012;24(4):363-368.
159. Wang SC, Shieh JF, Chang KY, Chu YC, Liu CS, Loong CC, et al. Thromboelastography-guided transfusion decreases intraoperative blood transfusion during orthotopic liver transplantation: randomized clinical trial. *Transplant Proc*. 2010;42(7):2590-2593.
160. Wikkelsoe AJ, Afshari A, Wetterslev J, Brok J, Moeller AM. Monitoring patients at risk of massive transfusion with Thrombelastography or Thromboelastometry: a systematic review. *Acta Anaesthesiol Scand*. 2011;55(10):1174-1189.
161. Johansson PI, Solbeck S, Genet G, Stensballe J, Ostrowski SR. Coagulopathy and hemostatic monitoring in cardiac surgery: an update. *Scand Cardiovasc J* . 2012;46(4):194-202.
162. Shore-Lesserson L, Manspeizer HE, DePerio M, Francis S, Vela-Cantos F, Ergin MA. Thromboelastography-guided transfusion algorithm reduces transfusions in complex cardiac surgery. *Anesth Analg*. 1999;88(2):312-319.
163. Girdauskas E, Kempfert J, Kuntze T, Borger MA, Enders J, Fassl J, et al. Thromboelastometrically guided transfusion protocol during aortic surgery with circulatory arrest: a prospective, randomized trial. *J Thorac Cardiovasc Surg* 2010;140(5):1117-1124.e1112.
164. Johansson PI. Coagulation monitoring of the bleeding traumatized patient. *Curr Opin Anaesthesiol* 2012;25(2):235-241.
165. Benes J, Zatloukal J, Kletecka J. Viscoelastic Methods of Blood Clotting Assessment – A Multidisciplinary Review. *Front Med (Lausanne)* . 2015;2:62.

166. Kaufmann CR, Dwyer KM, Crews JD, Dols SJ, Trask AL. Usefulness of thrombelastography in assessment of trauma patient coagulation. *J Trauma* 1997;42(4):716-720.
167. Cotton BA, Faz G, Hatch QM, Radwan ZA, Podbielski J, Wade C, et al. Rapid thrombelastography delivers real-time results that predict transfusion within 1 hour of admission. *J Trauma*. 2011;71(2):407-414.
168. Pezold M, Moore EE, Wohlauer M, Sauaia A, Gonzalez E, Banerjee A, et al. Viscoelastic clot strength predicts coagulation-related mortality within 15 minutes. *Surgery.* 2012;151(1):48-54.
169. Plotkin AJ, Wade CE, Jenkins DH, Smith KA, Noe JC, Park MS, et al. A reduction in clot formation rate and strength assessed by thrombelastography is indicative of transfusion requirements in patients with penetrating injuries. *J Trauma* 2008;64(2 Suppl):S64-68.
170. Doran CM, Woolley T, Midwinter MJ. Feasibility of using rotational thromboelastometry to assess coagulation status of combat casualties in a deployed setting. *J Trauma*. 2010;69 Suppl 1:S40-48.
171. National Health and Medical Research Council. Australian code for the care and use of animals for scientifice purposes. 8th Edition ed. Canberra: National Health and Medical Research Council; 2013.
172. Spahn DR, Rosaint R. Coagulopathy and blood component transfusion in trauma. *Br J Anaesth.* 2005;95(2):130-9.
173. Johansson PI, Sorensen AM, Perner A, Welling KL, Wanscher M, Larsen CF, et al. Disseminated intravascular coagulation or acute coagulopathy of trauma shock early after trauma? An observational study. *Crit Care*. 2011;15(6):R272.
174. Ho AM, Dion PW, Yeung JH, Holcomb JB, Critchley LA, Ng CS, Karmakar MK, Cheung CW, Rainer TH. Prevalence of survivor bias in observational studies on fresh frozen plasma and erythrocyte ratios in trauma requiring massive transfusion. *Anaesthesiology*. 2012;116(3):716-28
175. Mitra B, Wasiak J, Cameron PA, O'Reilly G, Dobson H, Cleland H. Early coagulopathy of major burns. *Injury*. 2013;44(1):40-3.
176. Maegele M. Coagulopathy after traumatic brain injury: incidence, pathogenesis and treatment options. *Transfusion*. 2013;53(Supp 1):28S-37S.
177. Mohr J, Ruchholtz S, Hildebrand F, Flohe S, Frink M, Witte I, Weuster M, Frolich M, van Griensven M, Keibl C, Mommsen P. Induced hypothermia does not impair

coagulation system in a swine multiple trauma model. *J Trauma Acute Care Surg* 2013;74(4):1014-20.

178. Torres LN, Sondeen JL, Ji L, Dubick MA, Torres Filho I. Evaluation of resuscitation fluids on endothelial glycocalyx, venular blood flow, and coagulation function after hemorrhagic shock in rats. *J Trauma Acute Care Surg*. 2013;75(5):759-66.
179. Martini WZ, Chinkes DL, Sondeen J, Dubick MA. Effects of hemorrhage and lactated Ringer's resuscitation on coagulation and fibrinogen metabolism in swine. *Shock.* 2006;26(4):396-401.
180. Fries D, Krismer A, Klingler A, Streif W, Klima G, Wenzel V, Haas T, Innerhofer P. Effect of fibrinogen on reversal of dilutional coagulopathy: a porcine model. *Br J Anaesth*. 2005;95(2):172-7.
181. Pragst I, Kaspereit F, Dorr B, Dickneite G. Prothrombin complex concentrate (Beriplex P/N) for control of bleeding after kidney trauma in a rabbit dilutional coagulopathy model. *Thromb Res*. 2010;125(3):272-7.
182. Grottke O, Braunschweig T, Philippen B, Gatzweiler KH, Gronloh N, Staat M, Rossaint R, Tolba R. A new model for blunt liver injuries in the swine. *Eur Surg Res*. 2010;44(2):65-73.
183. Martini J, Cabrales P, Fries D, Intaglietta M, Tsai AG. Effects of fibrinogen concentrate after shock/resuscitation: a comparison between in vivo microvascular clot formation and thromboelastometry*. *Crit Care Med*. 2013;41(11):e301-8.
184. Grottke O, Braunschweig T, Henzler D, Coburn M, Tolba R, Rossaint R. Effects of different fibrinogen concentrations on blood loss and coagulation parameters in a pig model of coagulopathy with blunt liver injury. *Crit Care*. 2010;14(2):R62.
185. Honickel M, Rieg A, Rossaint R, Braunschweig T, Spronk HM, ten Cate H, van Oerle R, Tolba R Grottke O. Prothrombin complex concentrate reduces blood loss and enhances thrombin generation in a pig model with blunt liver injury under severe hypothermia. *Thomb Haemost*. 2011;106(4):724-33.
186. Kheirabadi BS, Crissey JM, Deguzman R, Holcomb JB. In vivo bleeding time and in vitro thrombelastography measurements are better indicators of dilutional hypothermic coagulopathy than prothrombin time. *J Trauma*. 2007;62(6):1352-1359.
187. Klemcke HG, Delgado A, Holcomb JB, Ryan KL, Burke A, DeGuzman R, Scherer M, Cortez D, Uscilwicz J, Macaitis JM, Bliss J, Wojtaszczyk J, Christensen S, Currier H, Pusateri AE. Effect of Recombinant FVIIa in Hypothermic, Coagulopathic Pigs with Liver Injuries. *J Trauma*. 2005;59(1):155-61.

188. Martini WZ. The effects of hypothermia on fibrinogen metabolism and coagulation function in swine. *Metabolism*. 2007;56(2):214-21.
189. Park KH, Lee KH, Kim H. Effect of hypothermia on coagulatory function and survival in Sprague-Dawley rats exposed to uncontrolled haemorrhagic shock. *Injury*. 2013;44(1):91-6.
190. Iwamoto S, Takasu A, Sakamoto T. Therapeutic mild hypothermia: effects on coagulopathy and survival in a rat hemorrhagic shock model. *J Trauma* 2010;68(3):669-75.
191. Hatch Q, Debarros M, Eckert M, Satterly S, Nelson D, Porta R, Lesperance R, Long W, Martin M. Acute coagulopathy in a porcine venous hemorrhage and ischemia reperfusion model. *Am J Surg.* 2014;207(5):637-41.
192. Lesperance RN, Lehmann RK, Harold DM, Beekley AC, Sebesta JA, Martin MJ. Recombinant factor VIIa is effective at reversing coagulopathy in a lactic acidosis model. *J Trauma Acute Care Surg*. 2012;72(1):123-9.
193. Martini WZ DM, Pasateri AE, Park MS, Ryan KL, Holcomb JB. Does bicarbonate correct coagulation function impaired by acidosis in swine? *J Trauma*. 2006;61:99-106.
194. Darlington DN, Kheirabadi BS, Delgado AV, Scherer MR, Martini WZ, Dubick MA. Coagulation changes to systemic acidosis and bicarbonate correction in swine. *J Trauma*. 2011;71(5):1271-7.
195. Martini WZ, Pusateri AE, Uscilowicz JM, Delgado AV, Holcomb JB. Independent contributions of hypothermia and acidosis to coagulopathy in swine. *J Trauma*. 2005;58(5):1002-10.
196. Letson HL, Pecheniuk NM, Mhango LP, Dobson GP. Reversal of acute coagulopathy during hypotensive resuscitation using small-volume 7.5% NaCl adenocaine and Mg2+ in the rat model of severe hemorrhagic shock. *Crit Care Med*. 2012;40(8):2417-22.
197. Fung YL Tung JP, Foley SR, Simonova G, Thom O, Staib A, Collier J, Dunster KR, Solano C, Shekar K, Chew MS, Fraser JF. Stored blood transfusion induces transient pulmonary arterial hypertension without impairing coagulation in an ovine model of nontraumatic haemorrhage. *Vox Sang*. 2013;105(2):150.
198. Harr JN, Moore EE, Wohlauer MV, Droz N, Fragoso M, Banerjee A, Silliman CC. The acute coagulopathy of trauma is due to impaired initial thrombin generation but not clot formation or clot strength. *J Surg Res*. 2011;170(2):319-24.

199. Hayakawa M, Gando S, Ieko M, Honma Y, Homma T, Yanagida Y, Kubota N, Uegaki S, Sawamura A, Asakura H. Massive amounts of tissue factor induce fibrinogenolysis without tissue hypoperfusion in rats. *Shock*. 2013;39(6):514-9.
200. White NJ, Martin EJ, Brophy DF, Ward KR. Coagulopathy and traumatic shock: characterizing hemostatic function during the critical period prior to fluid resuscitation. *Resuscitation*. 2010;81(1):111-6.
201. Cho SD, Holcomb JB, Tieu BH, Englehart MS, Morris MS, Karahan ZA, Underwood SA, Muller PJ, Prince MD, Medina L, Sondeen J, Shults C, Duggan M, Tabbara M, Alam HB, Schreiber MA. Reproducibility of an animal model simulating complex combat-related injury in a multiple-institution format. *Shock*. 2009;31(1):87-96.
202. Mulier KE, Greenberg JG, Beilman GJ. Hypercoagulability in porcine hemorrhagic shock is present early after trauma and resuscitation. *J Surg Res*. 2012;174(1):e31-5.
203. Hagemo JS, Jorgensen JJ, Ostroswski SR, Holtan A, Gundersenn Y, Johannson PI, Naess PA, Gaarder C. Changes in fibrinogen availability and utilisation in an animal model of traumatic coagulopathy. *Scand J Trauma Resusc Emerg Med*. 2013;21:56.
204. Martini WZ. Fibrinogen availability and coagulation function after hemorrhage and resuscitation in pigs. *Mol Med*. 2011;17(7-8):757-61.
205. Egea-Guerrero JJ, Freire-Aragon MD, Serrano-Lazaro A, Quintana-Diaz M. Resuscitative goals and new strategies in severe trauma patient resuscitation. *Med Intensiva*. 2014;38(8):502-12.
206. Martin RS, Kilgo PD, Miller PR, Hoth JJ, Meredith JW, Chang MC. Injury-associated hypothermia: an analysis of the 2004 National Trauma Data Bank. *Shock*. 2005;24:114-8.
207. Eskens BJ, Leurgans TM, Vink H, Vanteeffelen JW. Early impairment of skeletal muscle endothelial glycocalyx barrier properties in diet-induced obesity in mice. *Physiol Rep*. 2014;2(1):e00194.
208. Bursa F, Pleva L. Anaerobic metabolism associated with traumatic hemorrhagic shock monitored by microdialysis of muscle tissue is dependent on the levels of hemoglobin and central venous oxygen saturation: a prospective, observational study. *Scan J Trauma Resusc Emerg Med*. 2014;22:11.
209. Bursa F, Pleva L, Maca J, Sklienka P, Sevcik P. Tissue ischemia microdialysis assessments following severe traumatic haemorrhagic shock: lactate/pyruvate ratio as a new resuscitation end point? *BMC Anesthesiol* 2014;14:118.

210. Desborough JP. The stress response to trauma and surgery. *Br J Anaesth*. 2000;85(1):109-17.
211. Haskins SC. Comparative cardiovascular and pulmonary effects of sedatives and anesthetic agents and anesthetic drug selection for the trauma patient. *J Vet Emerg and Crit Care.* 2006;16(4):300-28.
212. Burruss S, Andakyan A, Romanov S, Semiletova N, Cryer H. Effect of protein C gene mutation on coagulation and inflammation in hemorrhagic shock. *J Surg Res*. 2012;175(1):18-23.
213. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 2013;110(9):3507-12.
214. Reade MC, Kirkman E. The case for standardized reporting, registration, and a multicenter, multispecies approach to randomized controlled preclinical (animal) trials. In: Vincent JL (Ed). *Yearbook of Intensive Care and Emergency Medicine*. Berlin: Springer-Veriag; 2013
215. Lomas-Niera JL PM, Chang CS, Ayala A. Shock and Haemorrhage: an overview of animal models. Shock. 2005;24(Suppl 1):33-39.
216. Kiraly LN, Differding JA, Enomoto TM, Sawai RS, Muller PJ, Diggs B, et al. Resuscitation with normal saline (NS) vs. lactated ringers (LR) modulates hypercoagulability and leads to increased blood loss in an uncontrolled hemorrhagic shock swine model. *J Trauma*. 2006;61(1):57-64
217. Arnaud F, Hammett M, Philbin N, Scultetus A, McCarron R, Freilich D. Hematologic effects of recombinant factor VIIa combined with hemoglobin-based oxygen carrier-201 for prehospital resuscitation of swine with severe uncontrolled hemorrhage due to liver injury.*Blood Coagul Fibrinolysis* 2008;19(7):669-77.
218. Nishi K, Takasu A, Shinozaki H, Yamamoto Y, Sakamoto T. Hemodilution as a result of aggressive fluid resuscitation aggravates coagulopathy in a rat model of uncontrolled hemorrhagic shock. *J Trauma Acute Care Surg*. 2013;74(3):808-12.
219. National Health and Medical Research Council. Australian code for the care and use of animals for scientific purposes (8th Ed); 2013.
220. Charan J, Kantharia ND. How to calculate sample size in animal studies? *J Pharmacol Pharmacother*. 2013;4(4):303-6.
221. Brazzel C. Thromboelastography-Guided Transfusion Therapy in the Trauma Patient. *AANP J*. 2013;81(2):127-32.

222. McMichael MA, Smith SA. Viscoelastic coagulation testing: technology, applications, and limitations. *Vet Clin Path*. 2011;40(2):140-53.
223. Hauser CJ. Preclinical models of traumatic hemorrhagic shock. *Shock*. 2005;24(Suppl 1):24-32.
224. Tsukamoto T, Pape CH. Animal models for trauma research: What are the options? *Shock*. 2009;31(1):3-10.
225. Cox RA, Burke AS, Soejima K, Murakami K, Katahira J, Traber LD, et al. Airway obstruction in sheep with burn and smoke inhalation injuries. *Am J Respir Cell Mol Biol*. 2003;29(3 Pt 1):295-302.
226. Porada CD, Almeida-Porada G. Treatment of Hemophilia A in Utero and Postnatally using Sheep as a Model for Cell and Gene Delivery. *J Genet Syndr Gene Ther*. 2012;Suppl 1:011
227. Seekamp A, Dwenger A, Weidner M, Regel G, Sturm JA. Effect of recurrent endotoxemia on hemodynamics, lung function and neutrophil activation in sheep. *Eur Surg Res*. 1992;24(3):143-154.
278. Maybauer MO, Maybauer DM, Fraser JF, Westphal M, Szabo C, Cox RA, et al. Combined recombinant human activated protein C and ceftazidime prevent the onset of acute respiratory distress syndrome in severe sepsis. *Shock*. 2012;37(2):170-176.
229. Mujuni E, Wangoda R, Ongom P, Galukande M. Acute traumatic coagulopathy among major trauma patients in an urban tertiary hospital in sub Saharan Africa. *BMC Emerg Med*. 2012;12:16.
230. Baker SP, O'Neill B, Haddon W, Jr., Long WB. The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. *J Trauma* 1974;14(3):187-196.
231. Gennarelli T, Wodzin E. The Abbreviated Injury Scale 2005 - Update 2008. In: Barrington I, editor. *Association for the Advancement of Automotive Medicine* 2008.
232. Giannoudis PV, van Griensven M, Hildebrand F, Krettek C, Pape HC. Femoral nailing-related coagulopathy determined by first-hit magnitude: an animal study. *Clin Orthop Rel Res* 2008;466(2):473-480.
233. Lange M, Traber DL. Author's Reply. *Crit Care*. 2011;40:356-7.
234. Constable P. Fluid and electrolyte therapy in ruminants. *Vet Clin North Am Food Anim Pract*. 2003;19(3):557-97.
235. Stewart IB, McKenzie DC. The human spleen during physiological stress. *Sports Med* 2002;32(6):361-369

236. Turner AW, Hodgetts VE. The dynamic red cell storage function o the spleen in sheep. I: Relationship to fluctuations of jugular haematocrit. *Aust J Exp Biol Med Sci* 1959:37:399-420
237. Banks RE, Davis JA, Coulson NM, Beattie RJ. A paracostal approach for splenectomy in the sheep. *J Invest Surg*. 1988;1(2):143-8.
238. Mills LA, Simpson AH. In vivo models of bone repair. *J Bone Joint Surg Br*. 2012;94(7):865-74.
239. Archer R, Jeffcot L. *Comparative Clinical Haematology*. Oxford: Blackwell Scientific Publications; 1977.
240. Hruban R, Iacobuzio-Donahue C. Red Blood Cell and Bleeding Disorders. In: Kumar V, Abul K, Aster J, eds. *Robbins and Cotran Pathologic Basis of disease*. 9th ed: Elsevier; 2015: 629-67.
241. Xu L, Yu WK, Lin ZL, Tan SJ, Bai XW, Ding K, Li N. Chemical sympathectomy attenuates inflammation, glycocalyx shedding and coagulation disorders in rats with acute traumatic coagulopathy. *Blood Coagul Fibrinolysis*. 2015;26(2):152-60.
242. Xu L, Yu WK, Lin ZL, Tan SJ, Bai XW, Ding K, Li N. Impact of beta-adrenoceptor blockade on systemic inflammation and coagulation disturbances in rats with acute traumatic coagulopathy. *Med Sci Monit*. 2015;21:468-76.
243. Kutcher ME, Redick BJ, McCreery RC, Crane IM, Greenberg MD, Cachola LM, Nelson MF, Cohen MJ. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg*. 2012;73:13-9.
244. Simonova G, Tung JP, Fraser JF, Do HL, Staib A, Chew MS, et al. A comprehensive ovine model of blood transfusion. *Vox Sang*. 2014;106(2):153-160.
245. Mikulaschek A, Henry SM, Donovan R, Scalea TM. Serum lactate is not predicted by anion gap or base excess after trauma resuscitation. *J Trauma Acute Care Surg*. 1996;40(2):218-224.
246. Davis JW. The relationship of base deficit to lactate in porcine hemorrhagic shock and resuscitation . *J Trauma* 1994;36(2):168-172.
247. Iberti TJ, Leibowitz AB, Papadakos PJ, Fischer EP. Low sensitivity of the anion gap as a screen to detect hyperlactatemia in critically ill patients. *Crit Care Med* 1990;18(3):275-277.
248. Levraut J, Bounatirou T, Ichai C, Ciais JF, Jambou P, Hechema R, et al. Reliability of anion gap as an indicator of blood lactate in critically ill patients.*Intensive Care Med*. 1997;23(4):417-422.

249. Guyette FX, Meier EN, Newgard C, McKnight B, Daya M, Bulger EM, et al. A comparison of prehospital lactate and systolic blood pressure for predicting the need for resuscitative care in trauma transported by ground. *J Trauma Acute Care Surg*. 2015;78(3):600-606.
250. Kruse O, Grunnet N, Barfod C. Blood lactate as a predictor for in-hospital mortality in patients admitted acutely to hospital: a systematic review. *Scand J Trauma Resusc Emerg Med*. 2011;19(1):1-12.
251. Moomey CB, Jr., Melton SM, Croce MA, Fabian TC, Proctor KG. Prognostic value of blood lactate, base deficit, and oxygen-derived variables in an LD50 model of penetrating trauma. *Crit Care Med*. 1999;27(1):154-161.
252. Kastner SB, Kutter AP, von Rechenberg B, Bettschart-Wolfensberger R. Comparison of two pre-anaesthetic medetomidine doses in isoflurane anaesthetized sheep. *Vet Anaesth Analg*. 2006;33(1):8-16.
253. Borges LPB, Nishimura LT, Carvalho LL, Cerejo SA, Auckburally A, Mattos-Junior E. Behavioral and cardiopulmonary effects of dexmedetomidine alone and in combination with butorphanol, methadone, morphine or tramadol in conscious sheep. *Vet Anaesth Analg*. 2016 Feb 5 (epub ahead of print)
254. Dugdale A. Ruminants: Local and General Anaesthesia. *Veterinary Anaesthesia: Principles to Practice*. Oxford: Blackwell Publishing Ltd; 2010.
255. Hume ID, Sakaguchi E. Chapter 19: Patterns of Digesta Flow and Digestion in Foregut and Hindgut Fermenters. In: Sasaki Y, Kawashima R, editors. *Physiological Aspects of Digestion and Metabolism in Ruminants*. San Diego: Academic Press; 1991. p. 427-451.
256. Hagemo JS. Prehospital detection of traumatic coagulopathy. *Transfusion*. 2013;53 Suppl 1:48s-51s.
257. Yuan S, Ferrell C, Chandler WL. Comparing the prothrombin time INR versus the APTT to evaluate the coagulopathy of acute trauma. *Thromb Res*. 2007;120(1):29-37.
258. Owen CA, Jr. Historical account of tests of hemostasis. *Am J Clin Path*. 1990;93(4 Suppl 1):S3-8.
259. Eckman MH, Erban JK, Singh SK, Kao GS. Screening for the risk for bleeding or thrombosis. *Ann Intern Med*. 2003;138(3):W15-24.
260. Clouse LH, Comp PC. The Regulation of Hemostasis: The Protein C System. *N Engl J Med* 1986;314(20):1298-1304.

261. Ohlin AK, Larsson K, Hansson M. Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. *J Thromb Haemost*. 2005;3(5):976-982.
262. Conway EM, Van de Wouwer M, Pollefeyt S, Jurk K, Van Aken H, De Vriese A, et al. The lectin-like domain of thrombomodulin confers protection from neutrophil-mediated tissue damage by suppressing adhesion molecule expression via nuclear factor kappaB and mitogen-activated protein kinase pathways. *J Exp Med.* 2002;196(5):565-577.
263. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, et al. Dysfunction of endothelial protein C activation in severe meningococcal sepsis. *N Engl J Med*. 2001;345(6):408-416.
264. Tanaka KA, Fernández JA, Marzec UM, Kelly AB, Mohri M, Griffin JH, et al. Soluble thrombomodulin is antithrombotic in the presence of neutralising antibodies to protein C and reduces circulating activated protein C levels in primates. *Brit J Haematol*. 2006;132(2):197-203.
265. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. *Thromb Haemost*. 1991;65:618-623.
266. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng*. 2007;9:121-167.
267. Fu BM, Tarbell JM. Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. *Wiley Interdiscip Rev Syst Biol Med*. 2013;5(3):381-390.
268. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Archiv*. 2007;454(3):345-359.
269. Lipowsky HH. The endothelial glycocalyx as a barrier to leukocyte adhesion and its mediation by extracellular proteases. *Ann Biomed Eng*. 2012;40(4):840-848.
270. Davenport R. Pathogenesis of acute traumatic coagulopathy. *Transfusion*. 2013;53 Suppl 1:23s-27s.
271. Diggle P, Heagerty P, Liang K, Zeger S. Analysis of longitudinal data. In: 2nd Edition Oxford Statistical Sciences Series: Oxford University Press; 2002.
272. Frith D, Davenport R, Brohi K. Acute traumatic coagulopathy. *Curr Opin Anaesthes*. 2012;25(2):229-234.

273. Edul V, Enrico C, Laviolle B, Vazquez A, Ince C, Dubin A. Quantitative assessment of the microcirculation in healthy volunteers and in patients with septic shock. *Crit Care Med*. 2012;40(5):1443-1448.
274. Benninger E, Laschke M, Cardell M, Holstein J, Lustenberger T, Keel M, et al. Early detection of subclinical organ dysfunction by microdialysis of the rectus abdominis muscle in a porcine model of critical intra-abdominal hypertension. *Shock*. 2012;38(4):420-428.
275. Zambruni A, Thalheimer U, Coppell J, Riddell A, Mancuso A, Leandro G, et al. Endogenous heparin-like activity detected by anti-Xa assay in infected cirrhotic and non-cirrhotic patients. *Scand J Gastroenterol*. 2004;39(9):830-836.
276. Pierce A, Pittet J. Inflammatory response to trauma: Implications for coagulation and resuscitation. *Curr Opin Anaesthesiol*. 2014;27(2):246-252.
277. Kozek-Langenecker S, Sorensen B, Hess JR, Spahn DR. Clinical effectiveness of fresh frozen plasma compared with fibrinogen concentrate: a systematic review. *Crit Care*. 2011;15(5):R239.

CHAPTER 7: APPENDICES

7.1 Repeat statistical analysis of selected variables.

Variability in response was evident in the severe trauma animals. Animal 3 developed a higher blood lactate level and INR, longer aPTT and lower EXTEM A10 value than the other animals in the group (appendix 7.1.1). The impact of this variability on overall results was assessed by repeat statistical analysis of selected variables with this animal removed (appendix 7.1.2). A statistically significant difference in these variables remained, however the time at which statistical significance was achieved differed in some cases. The time at which the clinical definition of ATC was achieved was also prolonged (most notably for EXTEM A10) however all 3 proposed definitions were still met.

7.1.1 Individual responses of severe trauma group animals to selected variables.

The figures below demonstrate the individual responses of animals in the severe trauma to selected variables. Animal 3 demonstrated a greater rise in INR [A] and lactate [B] than the other animals in the group. A greater prolongation of aPTT [C] and reduction in EXTEM A10 values [D] was also demonstrated by animal 3 compared to the other animals in the group.

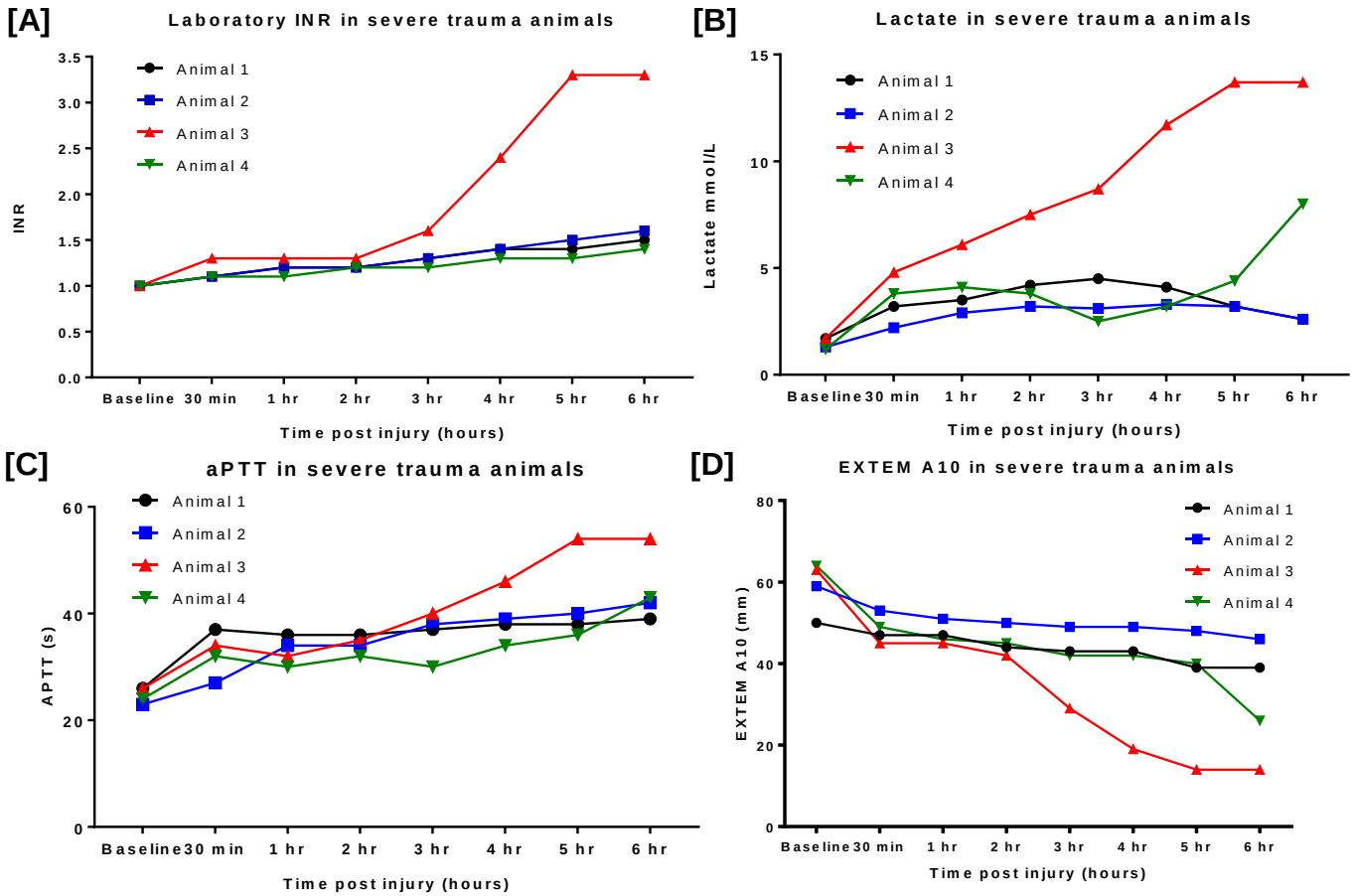

Figure 8. *Individual responses of animals in the severe trauma group to selected variables.*

7.1.2 Repeat statistical analysis of selected variables

Repeat statistical analysis of the selected variables presented above was undertaken to ascertain if a statistically significant difference in response remained without the inclusion of animal 3 (table 7). A statistically significant difference in all variables remained following the exclusion of animal 3 (severe (3)). The aPTT based definition of ATC was still met at 2 hours post injury (34.00 ± 2.00s) and a statistically significant difference (p =0.006) remained from 30 minutes post injury. However EXTEM A10 did not drop below 40mm until 6 hours post injury (37.00 ± 10.15mm) following the removal of animal 3, with a statistically significant difference (p=0.072) not evident until 5 hours post injury.

Table 7.*Repeat statistical analysis of selected variables following removal of severe trauma animal 3. Values are expressed as mean ± SD*

Variable	Baseline	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
aPTT (s)								
Control	24.25±1.50	23.50±1.92	23.25±1.89	24.00±2.00	24.50±2.08	23.75±2.88	24.25±2.50	24.75±2.50
Moderate	23.50±2.88	28.00±2.44	30.75±4.50^{x}	32.00±4.97^{x}	32.75±2.22^{x}	34.75±2.50^{x}	35.00±2.16^{x}	34.50±2.88^{x}
Severe	24.75±1.50	32.50±4.20*	33.00±2.58*	34.25±1.71*	36.25±4.35*	39.25±4.99*	42.00±8.16*	44.50±6.56*
Severe (3)	24.33±1.53	32.00±5.00*	33.33±3.01*	34.00±2.00*	35.00±4.36*	37.00±2.65*	38.00±2.00*	41.33±2.08*
INR								
Control	1.43±0.06	1.43±0.06	1.43±0.06	1.43±0.06	1.45±0.06	1.45±0.06	1.45±0.06	1.45±0.06
Moderate	1.33±0.06	1.38±0.10	1.48±0.06	1.48±0.06	1.55±0.06	1.63±0.06	1.65±0.06^{x}	1.65±0.06^{x}
Severe	1.33±0.10	1.35±0.06	1.38±0.10	1.43±0.06	1.48±0.10	1.60±0.20	1.73±0.32*	1.80±0.28*
Severe (3)	1.30±0.10	1.33±0.06	1.33±0.06	1.40±0.06	1.43±0.06	1.50±0.00	1.58±0.06*	1.68±0.06*
EXTEM A10 (mm)								
Control	61.00±6.06	55.75±4.44	54.75±4.58	52.00±5.94	53.00±5.72	51.75±8.34	54.00±6.80	52.75±6.60
Moderate	64.50±4.20	54.75±2.22	53.50±2.08	53.50±1.00	52.25±1.50	49.50±2.38	50.25±2.50	49.00±2.16
Severe	59.00±2.70	48.50±3.42	47.25±2.62	45.25±2.40	40.75±8.42*	38.25±13.2*	35.25±14.72*	31.25±14.2*
Severe (3)	57.67±7.09	49.67±3.05	48.00±2.65	46.33±3.22	44.67±3.79	44.67±3.79	42.33±4.93*	37.00±10.15*
Lactate (mmol/L)								
Control	1.23±0.38	0.58±0.20	0.53±0.20	0.53±0.16	0.55±0.20	0.50±0.14	0.55±0.14	0.55±0.09
Moderate	1.58±0.56	1.80±0.72	1.88±0.74	1.80±0.80	1.63±0.48	1.75±0.91	1.80±1.02	1.90±0.82
Severe	1.48±0.26	3.50±1.08	4.15±1.39*	4.68±1.93*	4.70±2.80*	5.58±4.10*	6.13±5.08*	6.73±5.30*
Severe (3)	1.40±0.27	3.07±0.81	3.50±0.60*	3.73±0.50*	3.37±1.03*	3.53±0.49*	3.60±0.69*	4.40±3.12*

Severe (3) = results with animal 3 removed, aPTT = activated partial thromboplastin time, INR = international normalised ratio, EXTEM A10 = clot amplitude at 10 minutes, * = significant difference between control and severe trauma groups p<0.05. x = significant difference between control and moderate trauma groups p<0.05

The original statistical analysis also demonstrated a significant rise in INR from baseline at 3 hours post injury in both the moderate (p=0.002) and severe (p=0008) trauma groups compared to the control group, with a 20% increase from baseline evident from 4 hours (figure 9 [A]). Following removal of severe trauma animal 3 the statistically significant increase in INR at 3 hours post injury in the severe trauma group compared to the control group was maintained (p=0.02). However the time at which the INR based definition of ATC was met was prolonged, with a 20% increased from baseline not evident until 5 hours post injury (figure 9 [B]).

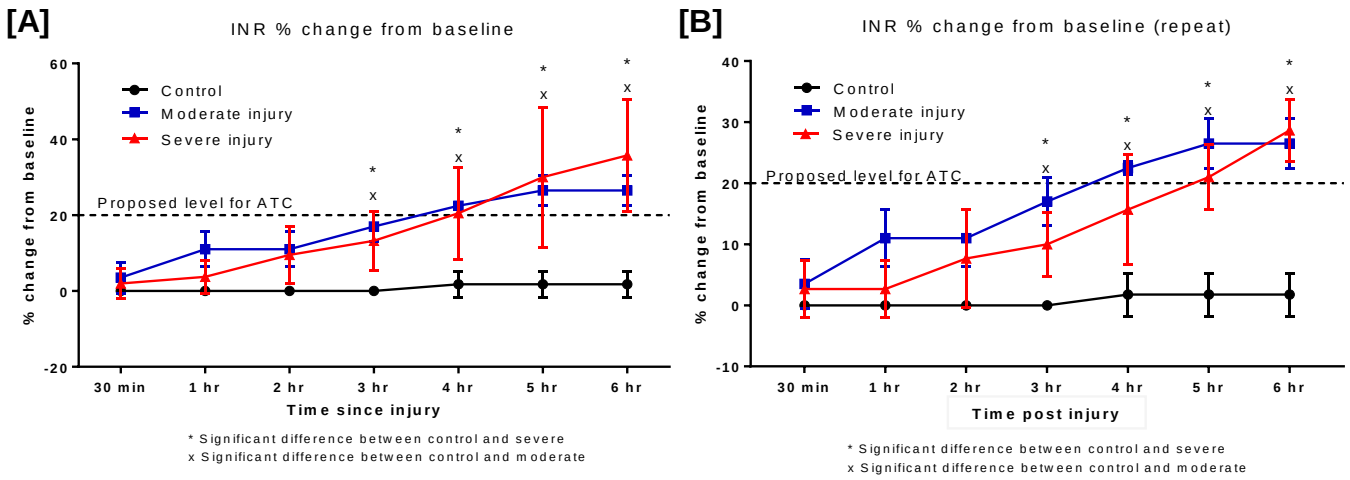

Figure 9. *INR changes in this thesis [A] An increase in INR was evident in the moderate trauma (p=0.002) and severe trauma (p=0.008) groups compared to the control group, with a 20% increase from baseline evident at 4 hours. [B] This increase was maintained in the severe trauma group following removal of animal 3 (p=0.02), however a 20% increase in baseline was not evident until 5 hours.*

7.2 List of manuscripts by the candidate included in the thesis

- van Zyl N, Reade MC, Fraser JF. *Experimental animal models of traumatic coagulopathy: A systematic review.* Shock 2015 44(1):16-24
- van Zyl N, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SG, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and haemorrhage*. J Trauma Acute Care Surg 2016; 81(4):674-684

7.3 List of published abstracts relevant to the thesis

- van Zyl N, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SG, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and haemorrhage* ANZ Journal of Surgery 2016;86 (sup 1): 162
- van Zyl N, Milford E, Diab S, Dunster K, Reade MC, Fraser J, Staib A. *Acute traumatic coagulopathy in an ovine model of trauma and haemorrhage.* Emergency Medicine Australasia 2015; 27 (sup 1): 51
- Milford EM, van Zyl N, Diab S, Dunster K, Tung, JP, Reade MC, Fraser JF. *The CHORuS study – using a large animal model of acute traumatic coagulopathy to test the efficacy of cryopreserved red blood cells compared to aged and fresh refrigerated red blood cells*. Journal of Military and Veterans Health, 2014, 23(1): 72-73.

7.4 List of oral presentations made by the candidate and relevant to the thesis

- van Zyl N, Milford EM, Diab S, Dunster K, McGiffin P, Rayner SG, Staib A, Reade MC, Fraser JF. *Activation of the protein C pathway and endothelial glycocalyx shedding is associated with coagulopathy in an ovine model of trauma and haemorrhage* The Royal Australian College of Surgeons (RACS) Annual Scientific Congress (ASC) 4th May 2016 Brisbane, QLD Australia

7.5 List of poster presentations made by the candidate and relevant to the thesis

- van Zyl N, Milford E, Diab S, Dunster K, Reade M, Fraser J. 2015. *A reduction in Factor VIII levels is associated with coagulopathy in an ovine model of trauma and haemorrhage*. Australasian Trauma Society Conference 2nd-4th October 2015, Gold Coast, QLD, Australia
- van Zyl N, Reade M, Fraser J, Collier C, Staib A, Bird R. 2015. *Coagulopathy in an ovine model of trauma and haemorrhage*. Princess Alexandra Hospital Annual Symposium 5th August 2015, Woolloongabba, QLD, Australia
- van Zyl N, Milford E, Diab S, Dunster K, Reade M, Fraser J, Staib A. 2014. *Validation of Acute Traumatic Coagulopathy in an ovine model of trauma and*

haemorrhage. Australasian College for Emergency Medicine (ACEM) Annual Scientific Meeting (ASM) 7-11th December 2014, Melbourne, VIC, Australia